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(54) Title: PORE-FORMING AND SUPERANTIGEN ENCODING TOXIN CASSETTES FOR GENE THERAPY

(57) Abstract

The present disclosure describes a segment of DNA which comprises a cytotoxin immunomodulatory gene which is modified for expression in eukaryotic cells and which genes are under the control of an inducible promoter. The disclosure also comprises a vector containing said DNA segment and a method of utilizing said DNA segment in cancer gene therapy.

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DESCRIPTI N
PORE-F RMING AND SUPERANTIGEN ENC DING TOXIN CASSETTES FOR GENE THERAPY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of gene therapy of tumors and more particularly to tumor therapy involving the insertion of toxin genes and immunomodulatory genes into tumor cells and controlling the expression of the toxin and immunomodulatory genes in the tumor cells by use of an endogenous or exogenously applied stimulus. In a certain embodiment, the invention relates to the induction of a "suicide response" in cancer cells through the application of an external stimulus which induces the expression of the toxin gene. The invention also relates to the use of nonenzymatically acting cytotoxic gene products that require synthesis of many molecules before a cytotoxic threshold level is reached in the cell, thereby lessening the incidence of side effects in human cancer gene therapy.

2. Description of the Related Art

Retroviral vectors have been used in a number of animal model systems for both gene therapy and anticancer therapy experiments. Examples of these are the gene transfer of the O6-alkyltransferase repair gene to enhance removal of adducts (Dumenco *et al.*, 1989), the transfer of cytokine genes, including TNF, IL2 and γIFN into tumor cells to stimulate tumor immunity (Gansbacher *et al.*, 1990; Gansbacher *et al.*, 1990), gene replacement therapy (Elitis *et al.*, 1985; Friedmann, 1989; Drumm

et al., 1990), and cellular immunization with TAR sequences against HIV infection (Sullenger *et al.*, 1990).

Highly lethal toxin proteins have evolved in plants and bacteria. Their mode of action depends on gaining entry into the cell by means of specialized ligand receptors at the cell surface. In the cell, these toxins either inhibit protein synthesis by cleavage of the rRNA molecules (*Shigella* and *Shigella*-like bacterial toxins, and the plant toxin, ricin) or disrupt protein synthesis by inhibiting poly (ADP) ribosylation of EF2 (Pseudomonas exotoxin and diphtheria toxin, DT). Another class of toxins are pore-forming toxins, like palyotoxins from marine multicellular organisms and the staphylococcal enterotoxins. The demonstration that diphtheria A chain (DTA) synthesis within the cell is equally as lethal as the whole toxin molecule endocytosed from the cell surface demonstrated the possible generation of a cell suicide response by a toxin transgene (Maxwell *et al.*, 1986). This paradigm has been applied to problems in developmental biology for tracing lineages (Palmiter *et al.*, *Science*, 1987, Palmiter *et al.*, *Cell*, 1987), and as an anticancer therapy strategy for tumor eradication (Maxwell *et al.*, 1986).

The major drawback of the class of toxins that consists of enzymatically acting protein synthesis inhibitors is their extreme lethality. DT-A chain, for example, is lethal at the level of one molecule per cell. This level of toxicity precludes the safe use of these toxins in transgenic therapy, which involves placing the toxin gene under the control of tissue/tumor-specific promoters, as these promoters invariably have low levels of basal activity (leakiness). The goal of specific tumor cell targeting has been to develop agents that can be designed to reach and recognize, or be specifically produced and/or active only in cancer cells (Raso, 1990).

Unfortunately, there was at the time of the present invention no method of expressing a toxin gene in tumor cells that overcomes the side effects due to constitutive low level activity in non-target cells.

Currently, the only approved therapies in humans involving targeting are based on ligand, or antibody recognizing specific tumor cell receptors or antigenic determinants (Byers *et al.*, 1989), and incorporate biomolecules such as naturally occurring toxins, monoclonal antibodies (MAbs) and biological response modifiers such as tumor necrosis factor (TNF), interferons (IFNs), and other cytokines, to provide the therapeutic effect. Despite promising *in vitro* results, *in vivo* targeting performance has been below expectations (Griffin *et al.*, 1988).

Besides imperfect target recognition, several drawbacks, including breakdown or modification of the agent in the blood, receptor-mediated clearance (especially from the liver), refractiveness by antigen modulation on tumor cells, and host immune response directed against the agent, all neutralize the therapeutic effectiveness of this approach. Although there is ample room to improve on this strategy by making more specific toxin-fusion proteins with ligands or antibodies exhibiting less non-specific binding and using factors to suppress host immune response (Ahmad and Law, 1990), this approach still presents the problem inherent with target design, that of non-specific toxicity to non-targeted tissue due to a lack of understanding of the target.

One of the promises of molecular genetic technology is that it is better suited to prevent problems of non-specific toxicity by producing a new class of "magic bullets" based on insight and knowledg of the genetic

working of the cell. The focus of this field of research r sts on identifying unique attributes of the target d tumor cell that are not present in all other normal cells, the basic premise behind "direct" targeting strategies. This immediately leads to the idea of using specific host-range modified viral vectors to transfect lethal genetic function into tumor cells or employing cancer/tissue specific promoter/enhancers to drive magic bullet genes. Although this should increase the accuracy of the targeting, unfortunately, it is only possible where there is a unique cancer cell determinant to exploit. However, for most human cancers, there is no specific handle of unique cell determinants to exploit for direct targeting.

One of the reasons these unique cell determinants have not been found is that cancers arise from a wide variety of physical, chemical, and infectious agents (Preston-Martin *et al.*, 1990). Tumors also exhibit a dynamic process of progression, in that variant subpopulations of heterogenous cells arise continually, often despite genetic evidence of a clonal origin, *e.g.*, initiation from a single cell (Nicolson, 1987). In addition, the specific molecular phenotype for each different type or grade of cancer is not known at present and presents a formidable research task to delineate (and is naturally complicated by progression and metastatic clones). Further problems arise due to chromosomal instability, which is a hallmark of cancer cells (Nowell, 1986). These genotypic changes lead to a diversification of phenotypes depending on microenvironments that are under various, not well understood selection pressures. Some of these selection pressures are epigenetically modulated and lead to the ability of cancer cells to metastasize (Trainer *et al.*, 1985). There is thus an immediate need f r an alternative strategy which can achieve the desired result of targeting specific tumor

cells, especially where targeting based on unique tumor-specific determinants is not possible.

SUMMARY OF THE INVENTION

The present invention seeks to overcome these and other drawbacks inherent in the prior art by presenting an alternative molecular genetic approach towards cancer cell targeting. Instead of deriving methods to target a killing response directly in tumors, which requires elucidation of cancer-specific markers/determinants/genetic programs, the present strategy entails defective retroviral mediated transduction of suicide genes into the proliferative compartment where the tumor resides in a reasonably controlled manner, and specific activation via promoters that respond to physical stimuli that is exogenously applied and targeted to the tumor cells. A defective retroviral vector is one of a class of replication-incompetent retroviruses, also termed self-inactivated (SIN) retroviruses, that has multiple mutations in the LTR that also abrogate transcription driven from the LTR's promoter. The suicide gene can lie dormant until activated, and ideally, from what is known with certain retroviral systems in replicative cells, have a limited time span before which, by some unknown epigenetic process, it is irreversibly inactivated (repressed).

Within the present disclosure, the term "suicide gene" refers to a dormant gene which exists within a cell and which, when expressed in that cell will cause the death of the cell in which it is expressed. The cytotoxicity may be caused by an interruption of the normal processes of cell growth or metabolism, or it may be mediated thr ugh the immune response of the host organism. Th terms "expressed" and "gene expression"

indicate, within the present disclosure, that a gene, or a region of DNA which encodes a polypeptide or peptide product is actively transcribed by an RNA polymerase and that the mRNA so produced is translated into a polypeptide or peptide molecule. This process preferably occurs within a cell or within the cells of an organism or within tumor cells, but the process can also occur in a cell free *in vitro* system such as, for example, a rabbit reticulocyte or wheat germ transcription/translation system.

An activated promoter, or an induced promoter, is a promoter region which is "turned on", or is actively promoting expression of the gene or genes which are under the control of said promoter. Within the present disclosure, the term "permeabilize" refers to the disruption of the integrity of the cellular membrane. This disruption allows small intracellular components such as, but not limited to, ATP, calcium ions, potassium ions and other usually charged small molecules, to leak out of the cell and may lead to a lack of viability in the cell.

One embodiment of the present invention is a genetic cassette, which cassette comprises a toxin gene which is modified to be expressed and translated in a eukaryotic host and which is under the control of a promoter sequence. In the present disclosure, the term "genetic cassette" refers to an intact stretch of DNA which comprises on either end a linker or polycloning region which contains, preferably, multiple sites for restriction enzyme digestion and which cassette can be removed from and/or added to other DNA sequences as a unit.

In a preferred embodiment of the present invention, the intact stretch of DNA would comprise a toxin gene and

preferably the gene enc ding a cytolytic enterotoxin which disrupts the plasma membrane integrity by generation of pore-forming complexes such as *Staphylococcus aureus* enterotoxin A (SEA). Five different strains of *Staphylococcus aureus* are known to secrete cytolytic enterotoxins. The *ent*-A gene product, SEA, is a water soluble, monomeric protein with broad reactivity towards membrane surfaces (Arbuthnot, *et al.*, 1973). The lipid bilayer is its primary target; binding to the membrane is accompanied by oligomerization of the 3S monomer to a membrane-bound 12S complex. In electron micrographs, the complex appears as a ring shape formation 8 to 10 nm in diameter, with a central 2-3 nm diameter pore (Fussle, *et al.*, 1981). The mode of action is analogous to immune cytolysis by complement, in that the SEA self-associates to form a pore that penetrates the lipid bilayer and permits the release of low molecular weight intercellular constituents.

The present invention also encompasses superantigen encoding toxins. The SEA toxin molecule is a bacterial superantigen which stimulates clonal proliferation of specific V_β T cell clones which result in the expansion of up to 4% of the T cell population. Because of this property it is the strongest mitogen for inducing cytotoxic T cell (CTL) response. In addition, this heightened mitogenic response by MHC Class II/SEA complex also involves increased activation of cytotoxicity and cytokine production (Herrman *et al.*, 1990). The SEA induction is correlated with a much greater binding affinity to class II MHC molecules (Bjorkman *et al.*, 1987). SEA-dependent cell mediated cytotoxicity (SDCC) results in the elimination of MHC Class II restricted SEA presenting target cells which can include tumor cells if they are engineered to present MSEA. In some instances the effectiveness of SDCC is not MHC Class II restricted.

It is contemplated that the modified SEA (MSEA) expression by the tumor and presentation on its cell surface might confer strong antitumor immunity by virtue of MSEA presentation to MHC class II pathway APC cells as a superantigen by immunization of the host with SEA or SEA-treated tumor membrane as the immunogen. This strategy circumvents the direct presentation by the tumor through MSEA peptide complexes with class I MHC molecules (the endogenous tumor pathway) which in most tumors is strongly down-regulated (reviewed in Hayashi *et al.*, 1985). In addition, the SE superantigens can elicit strong T cell response directly with the presentation of MHC class II-SE complexes. This pathway does not require antigen processing by antigen-presenting cells (APC). APC such as B cells, macrophages, dendritic cells, and a specialized subset of SEA reactive T cells (TCR-$(\alpha\beta)^+$ T cells) (Koning and Rust, 1992), can activate clonal expansion of cytotoxic T cells. It has also been shown that the targeting of tumor cells with SEA via conjugation with a tumor-specific monoclonal antibody (MAb) is effective not only against MHC class II positive tumor cells, but MHC class II negative cells by eliciting a strong CTL response and SDCC immunolysis (Dohlsten *et al.*, 1991; Hermann, *et al.*, 1991). Thus, MSEA expression on tumors could be an important way to bypass the problem associated with the deficiency of MHC class I presentation of "tumor antigens," observed as the immunogenic insufficiency to elicit tumor rejection when tumors are transplanted to syngeneic hosts (Hewitt *et al.*, 1976).

The present invention also encompasses the use of other superantigen encoding toxin cassettes which include, but are not limited to the staphylococcal enterotoxins B, C1, C2, C3, D, E and F; other staphylococcal-like enterotoxins such as streptococcal toxins and toxins of *Mycoplasma arthriditis*; and the

superantigens from mouse mammary tumor virus, for example the Mtv-7 superantigen (Winslow *et al.*, 1992) and other minor lymphocyte-stimulating antigens.

The toxin encoding gene of this embodiment may contain modifications that would allow the toxin to be expressed in eukaryotic cells, for example in yeast, animals or even humans and including human, rat or mouse tumor cells. The modifications could include, but are not limited to truncation of the gene at the first amino terminal residue of the active gene and the addition of a translational start and Kozak ribosome binding site consensus sequence upstream of the 5′ end of the gene, and for the 3′ end, termination and poly A signal sequences. Other internal modifications within the structural or active gene are also contemplated and would be included within the scope of the present invention (Marini, *et al.*, 1993). Such modifications would include, but are not limited to truncations, deletions, additions and substitutions of amino acids within the toxin which might affect the expression, toxicity or utility of the toxin. Such genetic manipulations are well known to those of skill in the art and would be encompassed by the present invention.

It is contemplated that the gene in the genetic cassette which encodes the toxin would also be under the control of a promoter which is expressible in a mammalian cell. Said promoters could include those which are isolated from mammalian or viral genomes, or any other source such as yeast. The promoters and enhancers that control the transcription of protein encoding genes in mammalian cells are composed of multiple genetic elements. The cellular machinery is able to gather and integrate the regulatory information conveyed by each element, allowing diff rent genes to ev lve distinct, often complex patterns of transcriptional regulation.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins. At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position n the same molecule of

DNA. This ability to act over a large distance had little precedent in classic studies of prokary tic transcriptional regulation.

Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities. They have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

There are two basic procedures for studying the *in vivo* expression of cloned genes and their promoters. In transient systems, the gene of interest (MSEA) is introduced into a population of cultured cells, and its activity is assayed within a few hours to a few days. The original transient expression experiments utilized encapsidated SV40 recombinants. Although only a small fraction of the cells take up and express the recombinant

genes, transcription of the foreign gene can be readily detected. Alternatively, if the promoter (control region) of the recombinant gene is under study, the promoter and enhancer can be cloned with the coding region of a gene such as Herpes Simplex thymidine kinase (tk) or *E. Coli* chloramphenicol acetyltransferase (CAT). The activity of the promoter can be monitored by an assay for the presence of the appropriate gene product.

The second method for studying cloned genes and their control regions is stable transfection. Stable transfection is the preferred method for obtaining moderate expression levels from a transfected gene in a long term continuous culture. In this method the recombinant DNA molecule (MSEA+promoter/enhancer combination) is introduced by DNA-mediated gene transfer techniques via viral infection. Identification of the recombinant stable transfectant among the population of untransformed cells requires a change in phenotype. Usually the inclusion of a drug selection marker aids in the discovery and selection of the stable transformants. Plasmids that are suitable for subcloning an expression cassette containing the MSEA sequence and any of the promoter/enhancer combinations listed below are well known to those of skill in the art. Such plasmids containing the MSEA sequence and promoter/enhancer can be used in a stable transfection protocol or transient transfection procedure.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the MSEA construct. Additionally any promoter/enhancer combination (AS PER the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of the MSEA transgene in a Gene Therapy protoc 1.

TABLE 1

ENHANCER	REFERENCES
Immunoglobulin Heavy Chain	Hanerji *et al.*, 1983; Gilles *et al.*, 1983; Grosschedl and Baltimore, 1985; Atchinson and Perry, 1986, 1987; Imler *et al.*, 1987; Weinberger *et al.*, 1988; Kiledjian *et al.*, 1988; Porton *et al.*, 1990
Immunoglobulin Light Chain	Queen and Baltimore, 1983; Picard and Schaffner, 1984
T-Cell Receptor	Luria *et al.*, 1987, Winoto and Baltimore, 1989; Redondo *et al.*, 1990
HLA DQ α and DQ β	Sullivan and Peterlin, 1987
β-Interferon	Goodbourn *et al.*, 1986; Fujita *et al.*, 1987; Goodbourn and Maniatis, 1985
Interleukin-2	Greene *et al.*, 1989
Interleukin-2 Receptor	Greene *et al.*, 1989; Lin *et al.*, 1990
MHC Class II 5^{k}_{α}	Koch *et al.*, 1989
MHC Class II HLA-DRα	Sherman *et al.*, 1989
β-Actin	Kawamoto *et al.*, 1988; Ng *et al.*, 1989
Muscle Creatine Kinase	Jaynes *et al.*, 1988; Horlick and Benfield, 1989; Johnson *et al.*, 1989a
Prealbumin (Transthyretin)	Costa *et al.*, 1988
Elastase *I*	Omitz *et al.*, 1987
Metallothionein	Karin *et al.*, 1987; Culotta and Hamer, 1989
Collagenase	Pinkert *et al.*, 1987; Angel *et al.*, 1987
Albumin Gene	Pinkert *et al.*, 1987, Tronche *et al.*, 1989, 1990
α-Fetoprotein	Godbout *et al.*, 1988; Campere and Tilghman, 1989

TABLE 1 (CONTINUED)

ENHANCER	REFERENCES
γ-Globin	Bodine and Ley, 1987; Perez-Stable and Constantini, 1990
ß-Globin	Trudel and Constantini, 1987
e-fos	Cohen *et al.*, 1987
c-HA-ras	Triesman, 1986; Deschamps *et al.*, 1985
Insulin	Edlund *et al.*, 1985
Neural Cell Adhesion Molecule (NCAM)	Hirsch *et al.*, 1990
α_1-Antitrypain	Latimer *et al.*, 1990
H2B (TH2B) Histone	Hwang *et al.*, 1990
Mouse or Type I Collagen	Ripe *et al.*, 1989
Glucose-Regulated Proteins (GRP94 and GRP78)	Chang *et al.*, 1989
Rat Growth Hormone	Larsen *et al.*, 1986
Human Serum Amyloid A (SAA)	Edbrooke *et al.*, 1989
Troponin I (TN I)	Yutzey *et al.*, 1989
Platelet-Derived Growth Factor	Pech *et al.*, 1989
Duchenne Muscular Dystrophy	Klamut *et al.*, 1990
SV40	Banerji *et al.*, 1981; Moreau *et al.*, 1981; Sleigh and Lockett, 1985; Firak and Subramanian, 1986; Herr and Clarke, 1986; Imbra and Karin, 1986; Kadesch and Berg, 1986; Wang and Calame, 1986; Ondek *et al.*, 1987; Kuhl *et al.*, 1987 Schaffner *et al.*, 1988

TABLE 1 (CONTINUED)

ENHANCER	REFERENCES
Polyoma	Swartzendruber and Lehman, 1975; Vasseur *et al.*, 1980; Katinka *et al.*, 1980, 1981; Tyndell *et al.*, 1981; Dandolo *et al.*, 1983; deVilliers *et al.*, 1984; Hen *et al.*, 1986; Satake *et al.*, 1988; Campbell and Villarreal, 1988
Retroviruses	Kriegler and Botchan, 1982, 1983; Levinson *et al.*, 1982; Kriegler *et al.*, 1983, 1984a,b, 1988; Bosze *et al.*, 1986; Miksicek *et al.*, 1986; Celander and Haseltine, 1987; Thiesen *et al.*, 1988; Celander *et al.*, 1988; Chol *et al.*, 1988; Reisman and Rotter, 1989
Papilloma Virus	Campo *et al.*, 1983; Lusky *et al.*, 1983; Spandidos and Wilkie, 1983; Spalholz *et al.*, 1985; Lusky and Botchan, 1986; Cripe *et al.*, 1987; Gloss *et al.*, 1987; Hirochika *et al.*, 1987, Stephens and Hentschel, 1987; Glue *et al.*, 1988
Hepatitis B Virus	Bulla and Siddiqui, 1986; Jameel and Siddiqui, 1986; Shaul and Ben-Levy, 1987; Spandau and Lee, 1988; Vannice and Levinson, 1988
Human Immunodeficiency Virus	Muesing *et al.*, 1987; Hauber and Cullan, 1988; Jakobovits *et al.*, 1988; Feng and Holland, 1988; Takebe *et al.*, 1988; Rowen *et al.*, 1988; Berkhout *et al.*, 1989; Laspia *et al.*, 1989; Sharp and Marciniak, 1989; Braddock *et al.*, 1989
Cytomegalovirus	Weber *et al.*, 1984; Boshart *et al.*, 1985; Foecking and Hofstetter, 1986
Gibbon Ape Leukemia Virus	Holbrook *et al.*, 1987; Quinn *et al.*, 1989

TABLE 2

Element	Inducer	References
MT II	Phorbol Ester (TFA) Heavy metals	Palmiter *et al.*, 1982; Haslinger and Karin, 1985; Searle *et al.*, 1985; Stuart *et al.*, 1985; Imagawa *et al.*, 1987; Karin ®, 1987; Angel *et al.*, 1987b; McNeall *et al.*, 1989
MMTV (mouse mammary tumor virus)	Glucocorticoids	Huang *et al.*, 1981; Lee *et al.*, 1981; Majors and Varmus, 1983; Chandler *et al.*, 1983; Lee *et al.*, 1984; Fonta *et al.*, 1985; Sakai *et al.*, 1986
ß-Interferon	poly(rI)X poly(rc)	Tavernier *et al.*, 1983
Adenovirus 5 E2	Ela	Imperiale and Nevins, 1984
Collagenase	Phorbol Ester (TPA)	Angle *et al.*, 1987a
Stromelysin	Phorbol Ester (TPA)	Angle *et al.*, 1987b
SV40	Phorbol Ester (TFA)	Angel *et al.*, 1987b
Murine MX Gene	Interferon, Newcastle Disease Virus	
GRP78 Gene	A23187	Resendez *et al.*, 1988
α-2-Macroglobulin	IL-6	Kunz *et al.*, 1989
Vimentin	Serum	Rittling *et al.*, 1989
MHC Class I Gene H-2kb	Interferon	Blanar *et al.*, 1989

TABLE 2 (CONTINUED)

Element	Inducer	References
HSP70	Ela, SV40 Large T Antigen	Taylor *et al.*, 1989; Taylor and Kingston, 1990a,b
Proliferin	Phorbol Ester-TPA	Mordacq and Linzer, 1989
Tumor Necrosis Factor	FMA	Hensel *et al.*, 1989
Thyroid Stimulating Hormone α Gene	Thyroid Hormone	Chatterjee *et al.*, 1989

The present invention also encompasses promoters which have been modified, by for example, truncation, deletion, insertion or site directed mutagenesis in order to render the promoter element more effective in the practice of the present invention. It is understood that the promoter would contain the control elements necessary to control gene expression in a mammalian cell and would comprise a "TATA" box region and any upstream elements necessary for RNA polymerase binding and expression in a mammalian host cell. The promoter of the present invention may also contain a combination of elements derived from more than one source and genetically combined.

Said promoter may also be an exogenously or endogenously controllable and inducible promoter that would respond to physical stimuli and would be adapted for this purpose. The promoters would include, but are not limited to such promoters as metallothionine promoters MtI and MtII which are inducible by heavy metals such as cadmium, and the heat-shock promoters from, for example hsp70A and B, and possibly promoters controlling DNA damage-inducible genes including X-ray or ionizing radiation inducible promoters such as the *Erg-1*,

c-Jun or TNF-α prom ters. The modification of such promoters through methods such as truncation, deletion, addition or substitution of nucleotide bases and selection of desired promoter function is well known to those of skill in the art and such modified promoters are within the scope of the present invention.

It is contemplated that in one embodiment of the present invention the toxin gene would be under the control of promoter elements which could be triggered externally by localized thermal or radiative energy in hyperthermia and radiotherapy. Since hyperthermia and radiotherapy constitute quite effective treatment modalities on their own, with the strategy of the present invention, additional therapeutic effectiveness is expected from these "molecular genetic" sensitizers.

The strategy of the present invention is an "indirect" targeting approach in which viral-mediated gene transfer of suicide transgenes encoding a toxin or toxic phenotype is placed under *ex vivo* control of an inducible promoter activated by certain physical stimuli. Targeting may be achieved by precise spatial localization of the inducing stimulus. This strategy requires that a foreign gene must be integrated into tumor cells, and remain dormant until activated by targetable inducing stimuli that are controllable by virtue of their physical nature.

With imaging techniques such as for example, X-ray computed tomography, positron emission tomography, and nuclear magnetic resonance imaging, accurate and high resolution morphological information on tumors and metastases is obtainable for the diagnosis of cancers. These techniques can become indispensable in the tr atment of the disease, if they are adapted to target the physical stimulus for a localized, pinpointed cell

killing or suicide response. It is possible to precisely target a physical stimulus such as heat or deposited ionizing radiation energy onto a tumor while sparing the surrounding tissue by close coordination with these imaging techniques. The stimulus is required to be selectively transduced to the effector elements of the gene and not be a natural component in homeostatic gene regulation in the cell.

Another embodiment of the present invention is a vector which comprises a modified double stranded DNA form of the vector sequence and the promoter/toxin gene cassette contained within the vector genetic sequence. The vector of the present invention is capable of being transferred into a cell and of integration of the vector genetic material containing the promoter/toxin gene cassette into the cell and preferably into the chromosome of the cell and furthermore stable expression through the vertical transmission of the gene. The vector would preferably be a retroviral vector and more preferably one from the pBABE series of retroviral vectors which give very high levels of constitutive expression by utilizing the 5′ LTR as a promoter (Morganstern and Land, 1990).

It is contemplated that the invention also includes, but is not limited to adenoviral vectors, adeno-associated vectors, a Herpes Simplex Virus vector, a minute virus of mice (MVM) vector, an Epstein-Barr Virus vector, a Simian Virus 40 vector as well as liposome vector systems. The vector may also be a DNA-ligand complex which permits targeting specific cells. Certain cell types can be distinguished by their expression of unique and restricted receptor patterns, and the ligands that bind these receptors can be used as carrier molecules to target the specific cells. The asiloglycoprotein receptor is found only on liver cells and on group (Wu and Wu, 1987) attached marker DNA via a

polylysine chain to the asiloglycoprotein for direct delivery to hepatocytes. Others have used ov rexpression of receptors for targeting marker DNA coupled with the ligand transferin (Wagner *et al*, 1991) or insulin (Rosencranz and Jans, 1992).

Another embodiment of the present invention is a method of treating human cancer patients which comprises the following steps.

a) Direct prophylaxis therapy. This form of therapy utilizes the cytotoxic property of MSEA or like toxin cassettes with a number of different vectors and/or delivery methods, the toxin gene would be introduced directly into the tumor by direct cannulization. As a secondary line of safety, the toxin gene could be controlled by cell-type-specific/cancer marker-gene-specific regulatory sequences (promoter/enhancer) or preferably by an *ex vivo* inducible promoter.

b) Antitumor immunization therapy. This form of therapy utilizes the superantigenic properties of MSEA or like toxin cassettes. After surgical debulking of the tumor, portions of the tumor would be grown out in culture, infected with the superantigen toxin containing vector, and then selected for expression of the superantigen toxin transduced gene. The superantigen toxin expressing cells would then be lethally irradiated and used to immunize the patient.

c) Superantigen targeted tumor infiltrated lymphocyte (TIL) therapy. Tumor infiltrated white blood cells (lymphocytes and macrophages) would be isolated from the surgically resected tumor. The macrophage component would be transduced with superantigen toxin to serve as an antigen-presenting cell for the lymphocyte T-cell fraction of the white blood cell component. Th

T-cells would then be activated and instructed by the superantigen-presenting macrophages, and once activated, would be expanded by traditional IL-2 therapy. These superantigen-activated TILs would be reinfused into the patient and would home back in on the tumor site.

d) A Combination of any of the above. Any of the above steps could be utilized as appropriate during the course of the therapy either individually or in combination with any of the other steps. The appropriate course of treatment would be determined by the practitioner for each individual patient.

Of course it is understood that the present invention is not limited to the clinical applications mentioned above, but has practical utility in a wide variety of applications including, but not limited to antibody screening for superantigen response, overproduction of antigens and bacterial proteins, studies of inducible promoters and evaluation of retroviral vectors. It is also understood that any method or tool for discovering new information about the mechanisms of the human immune response has potential for use in the development of new pharmaceutical agents and therapies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A. This figure illustrates the construction of the modified entA gene, MSEA. The sequences in this figure are designated SEQ ID NOS:1-2.

Figure 1B. This figure illustrates the restriction map of the resultant precursor in pSK II^+ verifying the correct structure and orientation.

Figure 2A. This figure shows a result of automated sequencing of the MSEA gene constructed by PCR mediated primer addition of appropriate signals on a truncated form of the SEA gene. The sequence in this figure is designated SEQ ID NO:3.

Figure 2B. This figure shows a second result of automated sequencing of the MSEA gene constructed by PCR mediated primer addition of appropriate signals on a truncated form of the SEA gene. The sequence in this figure is designated SEQ ID NO:4.

Figure 3. This figure shows the results of PCR analysis for viral titer and reverse transcriptase (RT) PCR for MSEA expression.

Figure 4. This figure shows a comparison of cytotoxicity of native SEA and intracellularly synthesized MSEA indicating that the intracellularly produced MSEA has comparable cytotoxicity to the externally administered SEA toxin.

Figure 5. This figure is the result of a Western blot study showing expression of the MSEA protein in two cell lines, human HT1080 and murine LF252.

Figure 6. This figure shows MSEA protein detected in membrane preparations of murine CT-26 cells. Lanes 1-4 contain cells treated with the following: Lane 1: naive CT-26, Lane 2: SEA coated (10 mg/ml) positive control, Lane 3: MSEA transfected (96 hrs.), Lane 4: Transfected with pBabeNeo (neg control). The cells after treatment were Parr bombed and membranes isolated by sucrose density gradient centrifugation. Lanes 5-9 show an increasing concentration of SEA (Sigma) as a standard.

Figure 7. This figure shows the measurement of ATP leakage out of cells by the permeabilization of the cells by SEA or MSEA. Murine CT-26 cells were treated with SEA (10 mg/ml) or lipofected (DOTAP-BMB) with MSEA minigene (96 hrs after treatment). ATP was measured by mixing supernatant with luciferin-luciferase and the luminescence was recorded.

Figure 8. This figure shows the increased cytotoxicity on cell populations treated with SEA or MSEA. Murine CT-26 cells were treated with either SEA (10 mg/ml) or transfected with MSEA minigene (96 hrs prior). The MTT dye (200 mg/ml) is reduced to a formazan precipitate that is extracted from the cells and spectrophotometriclly measured. Diminished dye conversion is correlated with cytotoxicity.

Figure 9. This figure shows tumor growth of a 30-day, *in vivo* transplantable tumor model. The weakly immunogenic murine cell line CT-26 was treated with no treatment (control) or coated with SEA(10 mg/ml). Forty eight hours later cells were harvested and $1x10^6$ cells were injected subcutaneously into the rear flank of a Balb/C mouse. Measurement of tumor area (in mm^2) were taken at a 10-day interval. Five animals were in each group.

Figure 10. This figure shows the result of tumor growth in a 30-day *in vivo* transplantable tumor model. The weakly immunogenic murine cell line CT-26 was treated by infection with pBabeNeo (control) or by infection with MSEA retrovirus (pBABEneoMSEA). Forty eight hours later, cells were harvested and $1x10^6$ cells injected subcutaneous into the rear flank of a Balb/C mouse. Measurement of tumor mass (in mm^2) was taken at 10-day intervals. Five animals were used in each group. The level of infectivity with this retroviral stock is on the order of 20%-30% of cells with a single infection.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A preferred embodiment of the present invention is the development of a toxin gene cassette designed for vector mediated insertion and expression in mammalian cells of a pore-forming and superantigen encoding molecule, termed MSEA, that has potential for anticancer gene therapy applications.

Recently, the NIH gene therapy and biosafety subcommittees have permitted limited use of retroviral vectors for human gene therapy in children suffering from severe combined immunodeficiency in which T cells are reinfused after retroviral insertion of a normal human ADA gene. The use of retroviral vectors was also approved in the case of TIL cells, in which retroviral insertion of a neomycin resistant (neo^r) drug selection marker is used in order to follow the homing and biological activity in tumors from humans with advanced melanoma. At the time of the present invention, direct gene insertion in humans is not an option until pioneering gene therapy experiments win acceptance for efficacy and safety.

The effective use of viral vectors depends on the receptor for the virus being present in the cell of interest. Fortunately it is known that most cell types are susceptible to retroviral infection (Donehower, 1987). Although tumor cell populations have the potential to rearrange or possibly antigenically repress these receptors, current data suggests that most tumor cell lines are also infectable but perhaps not all to a very high degree (Miller, 1990).

On potential problem with viral vectors is that the long terminal repeat (LTR) transcriptional regulatory

sequences have the potential of activating neighboring genes in cells, thus promoter insertion or insertional mutagenesis are side effects of random integration. In addition, inappropriate transcriptional activation from the LTR might override the transcriptional regulatory elements of the inserted gene. To circumvent this, self-inactivating (SIN) vectors which lack the viral promoter and/or enhancer element in the 3′ LTR control sequences, are used (Yu *et al.*, 1986). Since the 5′ LTR region of progeny viral particles arise from the 3′ LTR sequence, in the SIN vectors, the transcriptional activity of the LTR is nullified, and the inserted gene is transcribed only from an internal promoter.

Despite advances in packaging cell lines that are designed to produce only replication defective retroviral particles containing the recombinant sequences, recombination between exogenous functional genes with ubiquitous helper viruses, especially in murine and avian cells, may also lead to the problem of wild type viral production by recombination. However, this disadvantage of retroviral therapy is outweighed by the advantages.

The important advantage of using retroviral vectors in cancer therapy arises from the trait that retroviral vectors require replicating cells for productive infection, and without this replication, the integration and expression in the host genome does not take place (Miller, 1990). This, in principle, acts as a targeting mechanism in that only rapidly proliferating neoplastic cells would be the prime target, whereas slowly replicating regenerative cells in normal tissue and their terminally differentiated counterparts would be spared.

Another major advantage of using retroviral vectors is based on a common characteristic of all cancer cells. The evidence presented in a number of recent reviews on

the subject of human cancer (Preston-Martin, 1990; Ames and Gold, 1990; Cohen and Ellwein, 1990), shows that the common denominator is excess mitogenesis which can lead to all kinds of subsequent alterations in the genome and hence in the phenotypes of the cells. Thus the best strategy would appear to be to target proliferative cells rather than targeting cancer cells via specific determinants.

Normal tissues are composed of progenitor or stem cells that undergo replication and would therefore also be targets for retroviral insertion. However, retrovirally transduced genes that have been monitored for long term expression in gene replacement therapy situations reveal that, although the gene is stably integrated and not rearranged, for some presently unknown reason and by some epigenetic mechanism, the transgenes invariably become inactive with time (Palmer *et al.*, 1991). This, in essence, confers a limited life span in which a retrovirally inserted suicide gene in the cell poses a significant hazard, that is, the effect on somatic cells is self-limiting.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I

C nstructi n of MSEA T xin Cassett

The plasmid pMJB9 encodes the complete SEA protein, which is translated as a prepeptide and cleaved for translocation into active monomeric toxin subunits in bacteria (Betley *et al.*, 1984). In order to produce the active monomer for intracellular synthesis in mammalian cells, primer-mediated PCR modification of the gene was undertaken to truncate it at the first amino terminal residue (ser1) of the active monomer and to add translational start and Kozak ribosome binding site consensus sequences, and to add termination and poly A signals to the 3′ end (Fig. 1A-B). After PCR synthesis of the new gene, the modifications were confirmed by automated sequencing of the gel purified product (Fig. 2A-B).

The MSEA cassette was cloned into the pBABE series of retroviral vectors which give very high levels of constitutive expression by utilizing the 5′ LTR as a promoter (Morganstern and Land, 1990). The first effect that was noted was that the pBABE/MSEA plasmid when electroporated and selected on packaging cell lines was not as toxic as the diphtheria toxin A constructs, which invariably killed within 2-3 days. The lower toxicity of the MSEA construct permitted the production of retroviral stocks, however, by the end of the second week the effect of MSEA on packaging cell line viability was noted (Figure 4).

To confirm that virus was made from the packaging cell line, the supernatants were titered using the PCR method of Morgan (Morgan *et al.*, 1990), since a neo-titering method obviously would not be effective. This is shown in Fig. 3 for the RT PCR on the viral supernatants for BABE/MSEA, using both neo and SEA-

specific primers. This virus was then used to infect target cells and expression was monitored by RT PCR (cDNA amplification after reverse transcription of DNaseI treated RNA preparations) to assess whether the transcript was made. As shown in Fig. 3, the desired MSEA transcript is synthesized in the infected target cells. In figure 3, lanes 1 and 6 are low molecular weight markers. Lanes 2-5 exhibit the results of reactions using MSEA primers and lanes 7-10 are reactions with primers from the neomycin resistance gene. Lane 2 is the positive control for the presence of the MSEA sequence, viral supernatant MSEA amplimer, lanes 3 and 5 are the negative controls, uninfected LF252 hepatocarcinoma and LF252 infected with pGEN/ras-β-geo, respectively. Lane 4 is LF252 infected with pBABE/SEAneo and contains the DNA fragment of the correct size. Lanes 7 and 10 contain the positive controls for the presence of the neo gene sequence, viral supernatant amplimer and LF252 infected with pGEN/ras-β-geo, respectively. Lane 9 is LF252 infected with pBABE/SEAneo and contains the DNA product of the correct size.

The cells that are transfected with MSEA undergo a progressive decline over a 2-week period that is manifested as a ragged appearance of the cells in culture and the formation of patchy areas denuded of cells. This was confirmed by vital staining of the cultures with trypan-blue and observation of nonviable blue cells, over time. This is shown in Fig. 4. Titering was performed by incubating native SEA toxin for 1 hour in the concentration range of 0.1-10 μg/ml and comparing to increasing amounts of pBABE/MSEA plasmid introduced by cationic liposome transfection 48 hours previously.

Performing direct transfection with the plasmids counters th argument that nonspecific toxic products including MSEA made in the packaging cell lines and

liberated into the viral supernatant are the cause of the cytotoxic effect and proves specifically that gene transfer of MSEA is the cytotoxic principle. Using trypan blue as an endpoint for cell death, 10 μg/ml toxin induced approximately 100% cell death, while transient expression of MSEA with 5 μg plasmid produced greater than 90% cell death after 2 days. Though this suggests that a functional MSEA is the cause of progressive cell death, the inventors have sought to confirm this by other methods. Subcellular fractionation of the cells was undertaken and Western blots were prepared for analysis of MSEA location using a specific SEA monoclonal (Lapeyre, *et al.*, 1987) (Fig. 5).

For a superantigen encoding, pore forming toxin to function, it must be localized to a specific location within the cell. Pores must be generated in the plasma membrane, and additionally, for the correct presentation of the superantigen it must also be expressed on the external surface of the cell. Briefly, cells were transfected with MSEA and harvested at 4 days. $1x10^7$ cells were then dounce homogenized and subjected to N_2 cavitation via a Parr bomb. The resulting cell lysates were then separated on a sucrose step gradient (Clark *et al.*, 1989) and the membrane fraction isolated. These membrane fractions were subjected to SDS-PAGE (Laemmli *et al.*, 1970) blotted and probed with an antiSEA Ab (Lapeyre *et al.*, 1987). The resulting Western blot (Figure 6) shows Lane 1, naive CT-26; Lane 2, CT-26 cells coated with SEA (10 mg/ml); Lane 3, CT-26 transfected with MSEA; Lane 4, CT-26 transfected with pBabeNeo plasmid. The right hand panel shows increasing gradient of SEA in ng. Figure 6 demonstrates that the cells expressing MSEA show a correct band in the membrane fraction, very similar to the exogenously coated CT-26 cells, providing evidence that MSEA is synthesiz d within the cell and becom s localized in th plasma membrane.

The formation of pores on a cellular surface results in the escape of small intrac llular molecules. The release of small molecules, such as ATP, ions, etc., destabilizes the homeostatic mechanisms of the cell and leads to eventual cell death. The inventors measured the release of ATP from cells coated with SEA or cells expressing MSEA. Briefly, $1x10^6$ cells were coated with SEA (10 μg/ml) or lipofected with MSEA minigene (96 hr earlier). The media was removed and replaced with warmed PBS for 2 hrs. Next, the PBS was removed and an aliquot was quickly mixed with luciferin-luciferase and quantitated in a luminometer, as shown in Figure 7. Total cellular ATP is shown on the right and normal cellular loss is shown on the left. The SEA coated and MSEA expressing CT-26 shows a resultant loss of 55% to 75% of the total ATP, indicating permeabilization of the cell which results in intracellular content leakage.

Cell populations that express MSEA exhibit more cytotoxicity that their non-expressing counterparts. CT-26 cells that express MSEA and the resultant pore formation exhibit cytotoxic effects, due to leakage of their intracellular contents. This difference is measurable using a dye that is reduced in living, respiring cells. The dye, MTT, is converted to an insoluble blue formazan precipitate, which can be solubilized and measured spectrophotometrically (Mosmann, 1983). Briefly, $5x10^5$ cells were coated in SEA (10 μg/ml) and transfected with MSEA (96 hr earlier). MTT was added to the medium (final concentration 200 μg/ml), and after 4 hrs the cells were washed once in PBS- and the coverslips were immersed in 1 ml of isopropyl-HCL to extract the formazan precipitate. The remaining coverslip was then boiled in PCA and reacted with diphenylamine to quantitate the total DNA per coverslip. MTT absorbance was measured spectrophotmetrically at 570 nm and the values are corrected per mg f DNA. As shown

in Figure 8, cell populations coated with SEA (10 μg/ml) or transfected with MSEA (96 hrs earlier) exhibit 1 ss MTT conversion and are less viable.

In vivo studies of the cytotoxicity of MSEA and SEA, as indicated by tumorigenicity of CT-26 cells implanted in a syngeneic mouse recipient, are described in Figures 9 & 10. Briefly, $1x10^6$ CT-26 cells were coated with SEA (10 μg/ml) or infected with MSEA retrovirus ($2.7x10^4$ IU). Forty eight hours later the cells were harvested and injected subcutaneously into the hind flank of Balb/C mice. Five mice were used in each population group. The weakly immunogenic murine tumor CT-26 cells require approximately 21 days to grow to a palpable tumor mass. As shown in Figure 9 and 10, control tumors grow quickly and reach a maximal rate at 30 days (at which point they become necrotic and must be excised). The one-time SEA coated and MSEA transduced tumor cells showed reduced tumorigenicity in 5 animals. It is known that the *in vitro* retroviral infection protocol usually yields a conservative 20%-30% percent infection rate (using β-gal as a marker gene). It is contemplated by the inventors that a high intensity SEA or MSEA treatment protocol involving multiple applications and more highly efficient transducing viral vectors, AAV and adenovirus, for example, will result in even less tumorigenicity. In addition, it is contemplated that higher transduction efficiency may also yield higher cytotoxicity as the cells transduced with the MSEA toxgene are less viable.

This is a very important embodiment of the present invention, as the formation of an MSEA pore complex on the external cell surface of the plasma membranes would be expected to enhance therapeutic effectiveness of conventional chemotherapy by promoting the influx of chemotherapeutic drugs. Secondly, MSEA might change the tumor/host relationship by creating a strongly

immunogenic tumor for rejection that is not dependent on MHC class I antigen presentation. This requires presentation of the MSEA superantigen domain on the cell surface so that active immunization with sublethal SEA or a SEA/membrane immunogen might elicit antitumor CTL response.

EXAMPLE II

Retroviral Vectors for Gene Transfer

This embodiment of the invention has three components. The first component is plasmid cassettes harboring the neo^r drug-selectable marker and toxin genes from *Staphylococcus* A under the control of genetically engineered metallothionine I promoter. The second component is several viral vector preparations for the same toxin gene construct that are derived from different ecotropic and amphotropic packaging cell lines. The packaging cells are evaluated in order to determine the parameters associated with lines giving the most active viral titers by PCR assay described in Morgan *et al.*, 1990) and infectivity levels against the different malignant cell types, measured by either neomycin resistance and/or PCR assay for toxin gene insertion. With the establishment of malignant cell lines harboring a polyclonally integrated toxin gene, the third component is activation of the MT-driven suicide gene and determination of the kinetics and extent of cell killing by time-course measurements of the parameters associated with cell killing. These end-points are: (a) protein synthesis levels determined by immunoprecipitation of pulse-chase radiolabeled marker proteins; (b) cell and plasma membrane integrity by ^{51}Cr release assay; (c) loss of nuclear function revealed by chromatin fragmentation into oligonucleosome arrays; (d) cell death measured by vital and enzymatic fluorometric staining.

Construction of Toxin Gene Retroviral Vectors

Two typ s of retroviral backbones are used by the inventors and though not interchangeable with respect to applications, the constructs that are used depend in part on their clonability and expression levels. The retroviral backbones for inducible promoter expression are preferably pGen(-) obtained from Philippe Soriano (Soriano *et al.*, 1991), and pHMB obtained from Robert Hawley (Hawley *et al.*, 1989). Both backbones have microdeletions in U3R regions so as to be self-inactivating with respect to the promoter and enhancer at the 5'LTR end and thus amenable to expression from internal promoters. The EcoRI/HindIII fragment from pHMB has been deleted by T4 polymerase end polishing and XhoI phosphorylated linker addition so as to have the identical XhoI cloning site as found in pGEN-. This greatly simplifies the number of cloning steps for getting the promoter/toxin gene cassettes into vector constructs. The inducible promoter that is used is preferably a highly modified version of MT I promoter provided by Dr. Louis Ercolani (Ercolani *et al.*, 1990). This promoter is hyperinducible in the presence of 1-2 mM Cd ion and gives undetectable basal levels of expression. For constitutive levels of expression the pBABE series of retroviral vectors are used (Morganstern and Land, 1990).

Toxin Cassettes

Preferably the toxin cassette will contain a *Staphylococcus* enterotoxin gene. Five different strains of *Staphylococcus aureus* are known to secrete a cytolytic enterotoxin, called SEA when produced by the A strain. The toxin is a water soluble monomeric protein with broad reactivity towards membrane surfaces (Fussle *et al.*, 1981). The lipid bilayer is its primary target as binding to the membrane is accompanied by oligomerization of the 3S monomer to a membrane-bound 12S complex. In electron micrographs, the c mplex appears as a ring shape

formation 8 to 10 nm in diameter with a central 2-3 nm diameter pore. The mode of action is analogous to immune cytolysis by complement, in that the SEA self-associates to form a pore that penetrates the lipid bilayer and permits the release of low molecular weight intercellular constituents.

The cDNA encoding the entire *Staphylococcal* ent-A gene was generously supplied by Dr. Marsha Betley (Betley *et al.*, 1984). It is the B insert of pMJB2, a 2.5 kb HindIII fragment which is expressed in all *EntA*$^+$ strains. This HindIII fragment has been subcloned by the inventors into the HindIII site of Bluescript II SK to permit directional assembly of this cassette into retroviral vectors. The use of a protein toxin that requires the synthesis of multiple molecules for assembly into a pore complex offers a solution to the potential problem of inducible promoter leakiness. Thus, a basal level of SEA expression may be tolerated and only upon induction would there be sufficient amounts of SEA molecules to work as a suicide gene.

Packaging Cell Lines

The key component for using retroviral vectors to transduce foreign genes into recipient cells is the development of high efficiency packaging cell lines. These are designed to supply *in trans* all retroviral proteins necessary for assembly of an infectious retroviral vector particle. This enables a transfected modified viral vector sequence containing at the minimum the flanking LTRs in the extended packaging signal region (Ψ^+) to be transcribed and encapsulated to form an infectious viral particle that is replication defective (Watanabe and Temin 1983). Packaging cell lines have their problems associated with prolonged passage, including instability and possible recombination to

convert wild-type helper virus to contain the toxin gene.

Because suicide genes are placed into both murine and human tumorigenic cell types, various packaging cell lines are evaluated for viral production titers with the vector constructs, and for infectivity levels, especially for human cells which requires an *end* gene product supplied by an amphotropic packaging cell line. The two preferred packaging cell lines are based on each type's deletions and helper virus functions. These are ΨCRIP (amphotropic) and ΨCRE (ecotropic) packaging cell lines, obtained from Dr. Richard Mulligan. The ΨCRE line has a split coding region with mutations (Danos and Mulligan, 1988) and is known to give high titers, but is relatively unstable. The other preferred cell lines, obtained from Dr. Dina Markovitz, are Gp+E86 ecotropic cell line (Markowitz *et al.*, 1988) and Gp+EnvAM12, an amphotropic cell line (Markowitz *et al.*, 1988). The amphotropic cell line also has a split coding region, and provides *in trans* viral functions, but this line also contains further deletions to prevent regions of homology that would be capable of recombination and thus is considered to safer in regard to producing replication competent helper viruses.

Both of these cell lines allow for selection of the neo^r containing retroviral backbones (pGen and pHMB) which are used for vector production and establishment of latent toxin genes. These viral packaging cell lines are expanded and then frozen in multiple aliquots so that production is only allowed to take place over a limited time span and the cells are discarded after viral stocks are produced. Evaluation of vector production can be done by drug selection of the neomycin phosphotransferase (Tn5) gene with G418, in order to establish viral titers and titers based on the infectivity of transduction to

target cells. Viral titers in the range of 10^3 to 10^4 are expected and preferably in the range of 10^5 to 10^6.

Viral Stocks and Infections

After transformation and re-isolation of the retroviral plasmid construct, it is linearized at the *Dra*I site and introduced by electroporation into the packaging cell lines. Subconfluent packaging cells (1-2 x 10^6) are detached, washed in phosphate buffered saline (PBS) and electroporated with 20 μg of linearized DNA in 0.8 ml PBS using, for example a BioRad Gene Pulser at 290 V and capacitance of 480 μF as recommended by P. Soriano (Soriano *et al.*, 1991). Two days following electroporation, cells are selected in G418 (Gibco) at 1 mg/ml for 10 days giving polyclonal viral-producing cells. After replating in fresh DMEM supplemented with 10% fetal bovine serum, and shortly before reaching confluency, the virus is harvested from the media by filtration through a 0.45 μm filter. Recipient cells are seeded in T75 flasks and infected the next day with 1 ml of 1:10 dilution of virus in the presence of 4 μg/ml polybrene. After 2 days, cells are selected for neo^r for 10 days.

Titering

Recently, a rapid procedure has been described by Morgan *et al.*, (1990), which allows for rapid titering of virus and analysis for the integrity of the proviral structures in the transduced cells. In order to test viral stocks, the culture medium is precipitated with lithium chloride, reverse transcribed and then subjected to PCR amplification for either the neomycin or LTR regions and analyzed by gel electrophoresis which gives a relative titer of the virus produced. Using 200 ml of filtered medium, an equal volume of 8 M lithium chlorid is added and immediately frozen for a minimum of 1 hour at -20°C. Upon thawing, the RNA is pelleted at 14,000 x

g for 30 min. RNA is washed several times in 70% ethanol, dried under vacuum, and then reverse transcribed in: 33.5 ml of H2O, 2 ml of RNase inhibitor (1U/ml), 5 ml 10X RTase buffer (50 mM Tris HCl, pH 8.3, at 42°C, 75 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine), 5 ml of dNTP mix (20 mM for each dNTP) 2.5 ml 100 mM DTT, 1 ml 200 mg/ml random hexamers, and 1 ml AMV reverse transcriptase (10 U/ml). The reaction is incubated at 42°C for 1 hr, and then heat-inactivated at 70°C for 10 minutes. The entire reverse transcription reaction is ethanol precipitated and then subjected to PCR amplification conditions as described below.

The standard PCR reactions are set up with: 1-2 μg of genomic DNA, (10 picograms of viral DNA from media) in a reaction volume of 50 ml in PCR 1X buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 0.1% gelatin), 200 mM dNTP, 100 picomoles primers, and 2.5 U of ampliTaq polymerase. Each reaction is overlayed with 50 ml of mineral oil and subjected to 30 cycles of denaturation (94°C for 2 min), annealing (56°C for 2 min), and polymerization (72°C for 2 min) in an automated DNA thermocycler. After chloroform extraction to remove the mineral oil, samples are analyzed for diagnostic PCR products on a 4% polyacrylamide gel run in a 1X TBE buffer system and visualized under 300 nm UV light following staining with ethidium bromide. Oligonucleotide primers are synthesized preferably on a M380A Oligonucleotide synthesizer (ABI), and correspond to a region in the LTR spanned by primers, GTTTTCCAGGGTGCCCCAAGG (SEQ ID NO:5), and TGAGAGGAGACCCTCC (SEQ ID NO:6), which should give a 250 bp amplimer, and primers complementary to portions of the neomycin resistant gene CAAGATGGATTGCACGCAGG (SEQ ID NO:7), and GAGCAAGGTGAGATGACAGG (SEQ ID NO:8), which gives ris to a diagnostic 320 base pair amplimer.

Nuclear Fragmentation Assay

At designated times aliquots of cells are pelleted and lysed in 0.5 ml of buffer containing 0.1 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 8.0, 1% SDS and 50 mg proteinase K for 2 hours at 45°C. After phenol-chloroform extraction and ethanol precipitation, the DNA samples are treated with 20 mg/ml boiled RNase A in 1x TE for 30 minutes at 37°C and directly loaded on a 1.5% agarose gel or a 4% polyacrylamide gel for extensively fragmented samples (Chang *et al.*, 1989). Gels are calibrated with PM2 *Hae*III markers (0.1-2.0 kb) and nucleosome ladders produced by limited micrococcal nuclease digestion of nuclei (5-10% acid solubilization).

Protein Synthesis End-Points

The protein synthesis end-point is crucial for the determination of DT-A cytotoxicity, and might also be a parameter of interest in cell killing by SEA. Although SEA is known to induce the loss of cytoplasmic contents and disrupt the electrical potential of the cell membrane, the protein synthesis machinery is likely to be disrupted before overt cytolysis is discernable. This effect is determined by measuring the specific activity of newly synthesized proteins in pulse chase experiments and measurement of the incorporation of label into specific proteins of interest by immunoprecipitation.

An aliquot of cells (10^6) are placed in culture in methionine-free DMEM-1% fetal bovine serum for 30 minutes then labeled for 10 minutes with 100 mCi/ml [^{35}S]-methionine. The cells are washed in ice-cold PBS containing 10 mM methionine and lysed in 1 ml immunoprecipitation buffer (150 mM NaCl, 50 mM HEPES-OH, pH 7.4, 5 mM EDTA, 1% Triton X-100 and 5 mg/ml aprotonin/leupeptin mix added immediat ly before use) and centrifuged at 100,000 x g for 30 minutes. Protein concentration is determined by the Bradford assay. An

aliquot is taken for TCA insoluble cpm. For each immunoprecipitation assay, a minimum of 105 Cpm is incubated with excess antibody for 30 minutes and immune complexes are recovered by adsorption for 1 hour at 4°C on protein A-agarose beads, washed 4-5 times in 0.05 M HEPES-OH, pH 7.5, 0.1 M NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and solubilized in Laemmli loading buffer by heating at 95°C for 5 minutes (Furth *et al.*, 1982). The immunoprecipitated proteins are resolved on denaturing (SDS) polyacrylamide gels and visualized by ENHANCE fluorography. The specific proteins that are analyzed are control levels of actin filaments using the anti-actin MAb from Oncogene Sciences, proliferation-related oncoproteins anti-p67 c-myc MAb, anti-p62 c-fos MAb, anti-p105 RB MAb; and to monitor Ap-1 and PKC pathways polyclonal Ab against p39 c-jun and protein kinase C a,b,g isoforms (all from Oncogene Sciences).

Cytolysis Assay

Chromium labeled cells are prepared by incubating cells in a minimal volume containing 100 mCi $Na_2[^{51}Cr]_{04}$ followed by three washes with complete media. Release of ^{51}Cr is determined from radioactivity in cell-free supernatant at appropriate time-points. Extent of cytolysis is expressed as the percentage of total radioactivity released at each time-point.

Cell Viability/Cytotoxicity

The standard Trypan blue exclusion test for cell counting/viability is used to measure dead cells; however, because of the nature of the toxicity induced by SEA, which induces leakage of cytoplasmic contents, the two color fluorescence assay developed by Molecular Probes is used. This assay lends itself to quantitative analysis using image analysis software developed by Dr. Howard Gratzner and communicated to the inventors. This method permits following the process of induced cell

death by these two toxins in time course studies in which aliquots of cells at various time points are removed for end-point measurements. The LIVE/DEAD assay makes use of ethidium homodimer that is normally excluded from living cells and undergoes a fluorescent enhancement on binding DNA, while the counter stain is a pH insensitive fluorogenic esterase substrate, calcein-AM, which fluoresces green. The assay is a rapid, one step procedure that does not require removal of the dyes. It is performed, for example with a Zeiss Fluorescence microscope hooked up to a color recording video camera.

EXAMPLE III

Expression of Superantigen on the Cell Surface

Bacterial superantigens which are presented and bind extracellularly are exposed on the surface of the cellular membrane. Determining whether the superantigen epitope region encoded in MSEA is exposed extracellularly from the plasma membrane surface is accomplished by characterization of cell surface immunofluorescence and surface lactoperoxidase labelling studies using specific anti-superantigen epitope domain antibody made from a synthetic peptide. In addition, a functional assay for the presence of the superantigen epitope on the cell surface is conducted by *in vitro* ^{51}Cr cytotoxicity assays with effector cells from SEA immunized and non-SEA immunized allogenic mice against MSEA-expressing target cells, SEA-bound cells and unmodified target cells (test, positive and negative control, respectively) .

The biochemical characterization of the superantigen domain involves preparing a specifically pre-absorbed polyclonal antibody against the superantigen epitope on SEA by rabbit immunizations with a synthetic peptide (Griggs *et al.*, 1992, Grossman *t al.*, 1991). The pre-absorbed purified antibody is used for indirect

immunofluorescence studies on MSEA expressing and SEA-treated tumor cells using secondary mouse FITC anti-rabbit reagent. The anti-superantigen domain Ab is also used to immunoprecipitate surface labeled peptides using lactoperoxidase to catalyze iodination using the controls mentioned above to determine if the same immunoprecipitated labeled proteins are obtained as with SEA treated cells on Western blots. The antibody is also used in the functional assay in a blocking mode.

For the functional assay of the superantigen epitope in MSEA, *in vitro* cytotoxicity assays are performed with the leukocyte fraction obtained from Ficoll/Hypaque gradients of SEA immunized mice. ^{51}Cr release assays are performed with MSEA expressing versus non-MSEA expressing target CT26 cells to determine if CTL effector cells from immunized animals can be targeted against the MSEA expressing cells (Dohlsten *et al.*, 1991).

EXAMPLE IV
Testing Effectiveness of MSEA in Experimental Tumor Model Systems *In Vivo*

Recently, it has been shown that SEA superantigen targeted to tumor cell surfaces elicits a strong immune lysis response by CTLs against such tumors and this might be independent of Class I or II MHC expression (Dohlsten *et al.*, 1991; Hermann, *et al.*, 1991). In this embodiment of the present invention, the retroviral vector constructs incorporating MSEA toxin gene cassettes under control of specially modified promoters is evaluated for eradication of experimental tumors *in vivo*. The tightly regulated MTIIa promoter can be induced in 100% of the infected cells giving a theoretical maximum effect in cell culture, but this can also be accomplished in the animal by systemic activation by adding $CdCl_2$ to the animal's drinking water. Preferably, murine F10 melanoma

cells and the weakly immunogenic CT26 cells are infected without selection. To evaluate whether MSEA generates an antitumor immune response, SEA or SEA/CT26 plasma-membrane immunized groups of animals are challenged with MSEA-expressing and non-expressing CT26 tumor cells and tumor growth is monitored. The response is characterized in cyclosporin immunosuppressed mice and mice having selective $CD4^+$ and $CD8^+$ cells blocked by respective antisera.

Cell Lines

The highly metastatic F10 melanoma cell line obtained from Dr. Tatsuro Irimura and the CT26 cell line obtained from Dr. Phil Frost are utilized as experimental tumor models (Fearon *et al.*, 1990).

Animal model

For the B16/F10 cells, female (4-6 wk old) C57BL/6 are used, and given food and water *ad libitum*, and for CT26 cells, syngeneic Balb/c females of similar age are used. Viable tumor cells (10^6 CT26) dispersed in a single-cell suspension in 0.1 ml Earles basal salt solution (EBSS) is injected subcutaneously in the animal's flank. Subcutaneous tumor growth and spontaneous metastases are followed by periodic measurement of mean geometric diameter calculated as the cube root of the product of the maximum tumor length, width, and depth.

Organ Colonization

Tumor growth cells are assessed in the IV tail vein metastasis assay (Poste *et al.*, 1982). In the tail vein metastasis assay, 10^5 viable cells in 0.2 ml of EBSS cells are injected IV into the tail vein of unanesthetized mice. To activate MSEA expression, th host is dosed with 1 mM $CdCl_2$ f r systemic induction via the MTIIa promoter-driven MSEA gene. Visible tumor

colonization is appraised 21 days after injection or in moribund animals.

In Vivo Model for Antitumor Immunity

Whether or not MSEA expression by tumor cells can produce long term immunomodulatory effects and possibly produce an antitumor response is tested in weakly immunogenic CT26 cells (Fearon, *et al.*, 1990). CT26 tumor cells are modified by retroviral insertion of MTIIa/MSEA transgene as described above. Preferably, two groups of 5 animals are used in the initial pilot experiment. One group receives several immunizations at 3-week intervals of 0.1% of the LD_{50} of SEA (Sigma) in Freunds incomplete adjuvant IP until a demonstrable titer can be shown by ELISA. The LD_{50} is 40-60 mg/kg. This low level of SEA immunogen is used to preclude any gross toxic effects in the animals (Freer and Arbuthnott, 1983).

In one flank, untreated CT26 cells are implanted and compared with MSEA-expressing CT26 cells implanted on the opposite flank. This is performed in the two groups of mice: one that has been immunized with a sublethal dose of native SEA toxin, and one that has not. Although it is expected that MSEA expressing CT26 cells will grow more slowly and then eventually regress after considerable latency, if regression is much faster in the immunized mice, it is likely that MSEA was recognized as an antigen on the cell surface. The results are compared to the growth of MSEA expressing CT26 in nude mice versus non-immunized and immunized Balb/c mice.

Tumor Rechallenge Experiments

To establish that an antitumor immunity state has been reached, tumor rechallenge experiments are performed on animals having amputated regressing tumors. In one flank, 10^6 control CT26 cells infected with neomycin expressing virus are inoculated, while the opposite flank

is inoculated with the CT26 cells which have virally inserted MTIIa/MSEA integrated transgene. After tumor growth has reached a clearly measurable size (0.5 cm^3), the MSEA transgene is activated systemically by allowing the animals to drink water containing 1 mM $CdCl_2$. If the regression rate for the pre-immunized animals is significantly greater than for the non-immunized animals, it is likely that the host has mounted an immune response against the tumor by cytotoxic effector cells (CTL) recognizing MSEA as an antigen. In animals in which MSEA-expressing tumor is undergoing regression, the tumor is completely surgically amputated, and after a suitable recovery period, the host is rechallenged with either 10^6 MSEA expressing or non-expressing CT26 cells. This study determines whether tumor rejection is possible by commencing immunization <u>after</u> a tumor burden (0.5 cm^3) has been allowed to grow out. What is also determined is whether the tumor is subject to immune surveillance after rechallenge and if this requires representation of MSEA. The analogous study using the tail-vein I.V. assay is also conducted. This is accomplished in a disseminated metastatic melanoma model using B16(F10) cells that are induced to express MSEA after 5 days to allow for organ colonization in control versus SEA-immunized syngeneic hosts. After one month, or when animals appear moribund, autopsies are performed and the number and size of metastatic lesions in the lung, brain and liver tissues are quantified.

EXAMPLE V

Anticancer Gene Therapy

There are three theoretical ways in which the MSEA toxgene may be used for an anticancer gene therapy. The basic strategies are direct prophylaxis therapy, antitumor immunization therapy and superantigen tumor infiltrated lymphocyte (TIL) therapy.

Direct Prophylaxis

This is the most commonly contemplated approach for using a toxin gene to control cancer. Direct injection into the tumor site is the simplest way to get direct expression of the gene product within the cancer cells. This idea has been described by others, notably Ian Maxwell's group using the bacterial toxin, diphtheria A-chain gene, and George Moolten's proposed use of toxic phenotype genes. Huber *et al.* (1991) have made considerable use of these ideas with the *Varicilla zoster* virus (VZV) thymidine kinase (tk) gene for the treatment of liver cancer with the chemotherapeutic prodrug Ara-M; and a similar construct described by Blaese (Culvert *et al.*, 1992), using the herpes-tk gene delivered by a retrovirus to replicating tumor cells in the brain and killing these (tk^+) cancer cells by treatment with the prodrug ganciclovir. It is contemplated that the proposed use of MSEA would be similar to the Maxwell/Huber approach, in that the MSEA's gene product has direct therapeutic benefit when expressed specifically in the cancer cell. This may entail two levels of control to enhance the safety of this sort of approach for human therapy. The first requirement is the use of viral vectors that are designed to exhibit specific cell-type tropisms, e.g., are restricted to the cell types they can infect. In the case of retroviral vectors, their wide range of infectivity for replicating cell types poses certain advantages such as for brain tumors where the background normal tissue is non-dividing. In the case of employing direct DNA transfection modes such as ligand/DNA complexes, for example, the transferrin receptor (Wagner *et al.*, 1991) or asiloaloglycoprotein receptor to target the liver (Wu and Wu, 1988) the results are quite promising, but have not been extended beyond the liver to other cell types.

The second level of built-in safety is the use of specific regulatory lements to c ntrol the expression of the gene to the desired tumor cell type. For example, the alphafetoprotein gene is elevated in hepatocellular carcinomas and in cirrhotic liver or liver undergoing compensatory hyperplasia due to damage of toxicants, but it is not wholly expressed only in liver cancers. In the case of melanomas, the key gene controlling the melanin biosynthesis pathway, tyrosininse can be used to target melanomas, but it is expressed ubiquitously in all pigmented cells. Basically, the problem is that there is no absolutely specific cancer gene regulatory element that can be exploited. This leads to a compromised use of rather specific promoter elements that do not exhibit the kind of absolute specificity one would need to meet the very stringent safety requirement for use in humans. One way to circumvent this problem is to use prophylactic gene therapy agents that are less toxic so that side effects would not be the major problem. MSEA is very lethal yet it is much less cytotoxic than other toxgenes such as DTA and pseudomonas exotoxin (PE). Thus, the lack of absolute promoter specificity for the cancer cell and/or problems of basal promoter activity are not fatal to the use of MSEA as they are with DT-A.

Tumor immunization

Several important facets about superantigens make these entities attractive for tumor immunization. First, the superantigens, such as staphylococcal enterotoxins (SE), stimulate a very large repertoire of T-cells. The second feature is that this primed subset of T-cells (which is 1000 to 10,000-fold greater than a regular antigen presented by MHC class I or II molecules) are cytotoxic to cells presenting the superantigen. This killing is called superantigen directed cellular cytotoxicity (SDCC). The SE superantigen its lf is not presented conventionally in the groove of the MHC

molecule, but binds outside the groove and acts as a co-stimulatory ligand to the T-cell receptor (TCR). Recently, it has been demonstrated that tumors coated with SE molecules on their surface are rejected compared to the untreated tumors (Newell *et al.*, 1991). This rejection is not absolute however. This has been taken one step further and an immunotoxin has been created that carries the SE moiety to a cancer cell surface antigen targeted by the monoclonal antibody (Dohlsten *et al.*, 1991). The targeting of the SE to the cancer cell elicited SDCC and abrogated their growth. Secondly, and perhaps more interestingly, the SE presentation did not require the MHC class II molecules on the cell surface, e.g., could be used against MHC class II negative tumors. This is a very important point since previous attempts to immunize against tumors have been rather unsuccessful because the tumors in general down regulate expression of MHC class I and II molecules, thus limiting the presentation of regular antigens.

The fact that the SE superantigens elicit SDCC against MHC class II negative cells opens the door to employing these types of superantigens for antitumor immunization protocols. The lack of a co-stimulatory event required in superantigen activation of T-cells also allows the MSEA user to utilize dead cells in immunization. The use of immunotoxins, and in particular immunotoxins incorporating SE, are plagued with the common problem of clearance by the reticuloendothelial system, eventual elicitation of blocking antibodies and degradation of the immunotoxin rendering it non-specific in its effect. These problems are clearly overcome by developing an SE that can be expressed in mammalian cells and localized to the exterior portion of the cell membrane like the regular presentation with an immunotoxin or by direct cell surface adherenc .

The approach presented in the present disclosure makes use of these findings and carries them one step further with the development of a specially modified SE that is designed for intracellular expression in mammalian cells. This particular construct was developed for expression with a retroviral vector in tumor cells. The key points that have been demonstrated are that the SE is synthesized intracellularly, is localized both in the cytoplasm and plasma membrane, and exhibits cytotoxicity. The superantigen aspects of this construct are demonstrated in transgenic animals in which regulated expression takes place in MHC class II negative adult hepatocytes by placing the gene under major excreted protein (MEP) regulatory sequences and determination of what subset of T-cells targets the liver tissue.

Superantigen TIL Therapy

It has been demonstrated that TILs could be expanded *in vitro* with IL-2 and that these lymphocytes exhibited marked increased cytotoxicity against tumors from which they were isolated (Rosenberg *et al.*, 1991). IL-2 is a lymphokine that mediates the proliferation and activation of cytotoxic T-cells. However, cytotoxic T-cells that are expanded by IL-2 administration retain their restriction for the particular antigen that initially activated them, so in a population of TILs the relative proportion of tumor antigen-specific T-cells over non-tumor antigen specific T-cells remains the same. Therefore the clonal proliferation does not augment the relative number of tumor specific cytotoxic T-cells. Alternatively, a superantigen elicited mitogenic response in T-cells correlates with the activation of a subset of T-cells that are directed against specific antigenic determinants. With superantigen stimulation of TILs, the particular V_β subset that is stimulated with the superantig n presented by the tumor, is selectively activated and undergoes proliferation. Hence, the

relative proportion of specific cytotoxic T-cells against the tumor is augment d relative to the non-tumor activated T-cells found in that population.

In humans, superantigen proliferation enhances the expansion of about 4% of the T-cell subtypes bearing the particular V_β chain that is co-stimulated by the superantigen. It is expected that TILs isolated from tumors expressing the superantigen would be clonally expanded and represent the largest number of activated TILs from those isolated from the tumor. When these superantigen stimulated TILs are clonally expanded *in vitro* with IL-2, they are in much greater abundance than if they were not previously activated and expanded by stimulation with superantigens. For this type of therapy, the tumor that is treated by the direct prophylaxis approach is expected to generate a much larger relative number of antitumor cytotoxic T-lymphocytes in the TIL population than conventionally, and when isolated from the tumor and expanded *in vitro* would result in a much stronger antitumor TIL response than with the current protocol that does not involve superantigen stimulation.

Combination of the above therapies

It is clear from the discussion above that the maximum effectiveness of this approach would involve applying each of the above therapies at appropriate times during treatment. It is envisioned that where the tumor can be surgically excised, although in some cases not completely because of metastatic spread, the first line of therapy would be to infuse either systemically or in a localized manner, the therapeutic MSEA superantigen containing vector with the appropriate tropism and regulatory sequence for conditional expression in the tumor to obtain the highest degree of specific antitumor cytotoxicity. This therapeutic MSEA superantigen vector

would also be used to transfect MSEA into the tumor cells that would be cultivated *in vitro* for future antitumor immunization therapy as described. At the same time, the TILs and macrophages from the tumor could be co-cultured with these MSEA expressing tumor cells, and then expanded with IL-2.

The activity of the TILs may not be much greater than ordinary TILs isolated and expanded directly. However, during direct prophylactic treatment with the MSEA superantigen therapeutic vector, these TILs can be isolated, co-cultured with MSEA-expressing tumor cells to enhance their clonal proliferation and activation, and then expanded with IL-2 as described under superantigen activated TILs. It is expected that this will result in an extremely active TIL population directed against the tumor, and in particular, any metastatic variants that are likely to escape initial surgical resection and direct prophylactic treatment.

The reinfusion of TILs is a form of adoptive immunotherapy, wherein the source of the activated immunocompetent cells is derived from extracorporeal manipulation. Although this would be a very effective approach to total eradication of the tumor over time, it has the disadvantage that these TILs would lose their effectiveness, as tumor cells in the body are continually evolving and it would not be practical to maintain specialized TIL cultures for each individual for a protracted period of time (years). For this reason, it is much more feasible to also commence in the patient an antitumor immunization protocol toward the end of the TIL superantigen therapy to target the evolving tumor variants that are most likely to be picked up only by the patient's own immune system. This would entail periodic reinfusion or reimplantation f MSEA superantigen expressing radiation inactivated tumor cells. Becaus

they are killed and still serve as a strong present r f the MSEA superantigen as well as the tumor's own endogenous tumor antigens, they could be frozen down in aliquots and reinfused on a periodic immunization schedule to boost the antitumor response and maintain it over a long period of time.

EXAMPLE VI

Heat and Ionizing Radiation Activated Promoters

In mammalian cells, heat shock protein (hsp) gene products mediate a protective effect against heat and have recently been linked to enhanced viral reactivation which is a response to DNA damage (Pelham, 1985). A cellular heat shock transcriptional protein, HSTP, is the mediator of the effect in heated cells by a change in conformation that can be mimicked by antibody binding (Wu, 1984). The active conformation interacts with the heat shock element (HSE) (Pelham, 1982). The HSE are multiple cis-acting elements in eukaryotic heat shock promoters, that have no simple consensus sequence but a basic motif possessing a hyphenated dyad axis, C-GAA/TTC-G (Pelham, 1985). Despite different thermal sensitivities in *Drosophila* (25-37°C) and in man (37-42°C) the HSE from *Drosophila hsp*70 promoter works efficiently for activation in human cells by its HSTP so that the heat shock stimulus is transduced within minutes to activate transcription. It is a transient response since a return to normal conditions turns it off. The effect of heating is also manifested by translational arrest and the preferential translation of the heat shock-induced mRNAs (Mariano and Siekierka, 1986). These salient features provide distinct advantages for employing heat-shock vectors.

Several vectors have been designed around the *Drosophila hsp*70 promoter and have been exploited f r

the expression of *c-myc* (Warm *et al.*, 1986) or HIV TAT gene (Schweinfest *et al.*, 1988), in a controlled manner in human cells. Although the *Drosophila hsp*70 promoter has been placed in vectors and claimed to be tightly regulated, e.g., expression is not detected at 37°C (Warm *et al.*, 1986; Knipple and Marsella-Herrick, 1988), the high degree of homology it shares with the *hsp*70A promoter from humans, cloned by Hunt and Morimoto (Schweinfest *et al.*, 1988), might be problematical as the *hsp*70A promoter is regulated by other pathways, including serum replacement after starvation via the serum response element (SRE) at -48, heavy metal induction like MT promoters, viral induction (Hunt and Morimoto, 1985), in addition to LPS, γIFN, and c-myc also triggering *hsp*70A induction. These, in the context of driving toxin genes, would be very undesirable from the standpoint of their response to physiological stimuli. Inappropriate triggering not related to heat would make the engineering of the *hsp*70A promoter or utilization of *Drosophila hsp*70 promoter very important. In the *hsp*70 gene family, the *hsp*70A promoter is quite different from another promoter cloned from the *hsp*70B gene by Voellmy (Voellmy *et al.*, 1985). The *hsp*70B promoter has been shown to exhibit basal levels of expression in certain cell types (Mivechi and Rossi, 1990), but is amenable to having the leakiness problem ameliorated which would render the promoter safe to use in conjunction with MSEA.

The *hsp*70B promoter contains a single degenerate HSE element rather than the four identified in *hsp*70A. The major difference is that this is a highly GC rich promoter containing 5 GC boxes for the interaction with SP1 and a number of other elements, which were defined by a computer search of cis-acting consensus sequences (Delaney Software) and might possibly interact with the *hsp*70B promoter. The sequence organization of the *hsp*70B promoter is quite similar to that of constitutively

expressed housekeeping genes, although as mentioned previously, it is tightly regulated in certain cell types, HeLa for example (Voellmy *et al.*, 1985), but apparently is expressed constitutively in others (Mivechi and Rossi, 1990). The inventors have mapped the minimal core *hsp*70B promoter by successive truncations starting in the furthermost upstream region of the insert in p17 (Voellmy *et al.*, 1985). The region spanning the *Hind*III to *Sma*I sites was found to form a minimal promoter of 225 base pairs in length. In transient expression assays in CV1 cells the core promoter was strictly heat inducible at 42°C, and not triggered by other agents known to affect *hsp*70A expression, including calcium ionophore, phorbol ester (TPA), LPS, γ-IFN, and serum replacement. It is contemplated that similar results may be obtained in other cells in which it is claimed that the intact *hsp*70B gene is turned on and expressed at basal levels. The *hsp*70B gene also differs with the *hsp*70A promoter in that it does not contain a TATA sequence, which in the *hsp*70A promoter interacts obligatorily with TFIID, thus making it responsive to E1A transactivation (Simon *et al.*, 1988). Thus the B promoter is not inherently susceptible to viral inductions.

Ionizing radiation inducible promoters may also be used as an embodiment of the present invention. Such promoters include, but are not limited to the *Egr-1* promoter, the *c-Jun* promoter, the tumor necrosis factor-alpha (TNF-α) promoter or promoters "trapped" by the method of Example VIII, *infra*. The *Egr-1* promoter region contains several putative cis elements including six CArG domains ($CC(A/T)_6GG$) (Christy, *et al.*, 1989; Qureshi, *et al.*, 1991). The CArG region has also been shown to be required for *c-fos* induction by both PKC-mediated (protein kinase C) signaling pathways and by growth factor induced signals independent of PKC (Fisch, *et al.*, 1987; Gilman, 1988). The *c-Jun*/*c-fos* gene families

encode transcription factors and are known to be activated by exposure to X-ray radiation (Hallahan, *t al.*, 1991; Sherman, *et al.*, 1990). TNF-α is a polypeptide mediator of the cellular immune response with pleiotropic activity. TNF-α causes hydroxyl radical production in cells sensitive to killing by TNF-α (Matthews, *et al.*, 1987.) Cell lines sensitive to the oxidative damage produced by TNF-α have diminished radical buffering capacity after TNF-α is added (Yamauchi, *et al.*, 1989.) As with the metallothionein and heat shock promoters, it is contemplated that these or other ionizing radiation inducible promoters may be engineered to find those regions which respond to the ionizing radiation and which will afford the tightest control of basal expression.

The engineering of the promoters involves identifying motifs that control basal levels of activity and, by subsequent sequence modification or deletion, optimizing promoter function to obtain low basal levels. Even at low basal levels, leaky toxin expression, especially of DT-A, would compromise the effectiveness of this embodiment by provoking uncontrolled cell suicide. Therefore, less lethal toxin genes are used, such as the gene for the SEA pore-forming protein. Secondly, it is necessary to determine what other physiological stimuli and through what pathway these promoters may be activated. Once these are determined, these elements are deleted in order to prevent the problem of non-specific triggering. The standard for comparison of the degree of regulation is a genetically engineered version of metallothionine promoter I obtained from Louis Ercolani. The metallothionine promoter is the prototypic example of an extensively modulatable promoter that has been engineered for a high level of inducibility, yet it cannot be used in the immediate embodiment because its activati n, though externally controlled by heavy metals,

is a systemic, non-targeted response. It is, howev r an ideal promoter to study the strategy with malignant cells that are pre-infected with the suicide gene, then transplanted in the host for *in vivo* eradication experiments. Because it can be systemically activated by Cd ions, it will be compared against the spatially controllable and modulatable promoters.

EXAMPLE VII

Heat and Radiation Activation of Toxin Genes in Tumor Cells

Vector constructs incorporating MSEA toxin genes under control of specially modified promoters are evaluated for eradication of experimental tumors. In order to compare the efficacy of the induced suicide response, expression of toxin genes under control of the MT I promoter is compared to expression of the same genes under control of heat inducible promoters, using MSEA toxin genes. The tightly regulated MT I promoter can be induced in 100% of the infected cells giving a theoretical maximum effect in cell culture and in the animal by systemic activation by administering $CdCl_2$ in the animal's drinking water. It is contemplated that there may be some synergistic effect with the heat or radiation activated promoters and this might effect the theoretical maximum. The efficacy of the toxin cassettes is first tested in cell culture wherein the conditions for induction can be more precisely controlled and the end-points more precisely measured. Then, the best responding regulated promoter, for example the modified *hsp*70B, or the ionizing radiation responsive DDI promoter, or the ionizing radiation sensitive promoters discussed in Example VI *supra*, is evaluated in the tumor model transplanted *in situ*. In this model system, the cells are pre-infected. This is to test the delivery and specificity of the targeting of the inductive stimulus in

the intact animal, and to test its efficacy at tumor eradication *in vivo*.

Animal Model

Six-week old female Balb C nude/nude mice weighing 20-25 grams are maintained in a closed colony with autoclaved food and cage materials, and are used with the human K1795 melanoma cells. For the B16/F10 cells, female (4-6 wk old) C57BL/6 are used, and given food and water *ad libitum*. Viable tumor cells (10^6) dispersed in a single-cell suspension in 0.1 ml Earles basal salt solution (EBSS) are injected subcutaneously in the foot pads of the animals (Trainer *et al.*, 1985; Horsman *et al.*, 1990). The animals are observed on a daily basis to monitor any impairment due to tumor growth according to institutional animal care guidelines. Subcutaneous tumor growth and spontaneous metastases are followed by periodic measurement of mean geometric diameter calculated as the cube root of the product of the maximum tumor length, width, and depth. When the tumors reach diameters greater than 2 cm, the mice are anesthetized, and tumors surgically removed along with a 2-3 mm margin of normal tissue. Animals moribund from tumor growth or metastatic progression, are anesthetized and organs and tumors removed aseptically.

Hyperthermia Treatment

Mice are placed in lucite restraining holders that have holes in the bottom allowing for immersion of the feet approximately 1 cm below the water surface. Tumor bearing feet are immersed in a circulating water bath stabilized at the desired temperature, calibrated against a certified thermocouple (Horsman *et al.*, 1990). It has been empirically determined that the temperature of the water should be adjusted to about 0.2°C above the desired tumor temperature. Treatment duration is based on time

and temperature values determined for maximum effectiveness during cell culture studies.

Ionizing Radiation Treatment

In a certain embodiment of the invention, tumor cells which contain the toxin cassette under the control of a radiation inducible promoter may be exposed to an effective dose of ionizing radiation. The radiation could be produced by, for example a 250 kV x-ray generator. It is contemplated that an effective amount of radiation would be preferably from about 2 to about 20 Gray administered at a rate of from about 0.5 to about 2 Gray per minute. More preferably an effective amount would be from about 5 to about 15 Gray.

Organ Colonization

Tumor growth and implants in various organs are assessed following either the IV tail vein assay, or the subcutaneous injection of tumor cell suspensions, free of clumps, into the foot pads (Trainer *et al.*, 1985; Horsman *et al.*, 1990). In the tail vein metastasis assay (Poste *et al.*, 1982), 10^5 viable cells in 0.2 ml of EBSS cells are injected IV into the tail vein of unanesthetized mice. Visible tumor colonization is appraised 21 days after injection or in moribund animals. Interorgan colonization is detected under the dissecting microscope by the presence of pigmented focal lesions. These are scored for number and location in regional lymph nodes, lungs, liver, brain, and sporadic metastases to other organs such as ovaries.

EXAMPLE VIII
Isolation of Inducible Promoters

In another embodiment of the present invention, inducible human promoters can be identified and is lated for use in a toxin gene cassette, or for use in any

number of human genetic therapies. Preferably, this is accomplished by retroviral mediated transfer of a promoterless marker gene with an upstream universal splice donor/acceptor site that can integrate anywhere within intronic or exonic regions of a gene and, with proper selection, be screened for the appropriate functional activity promoter. To monitor expression, the ideal reporter gene would be one that allows for cell sorting based on expression under inducible control of its promoter. Recently, Herzenberg's group has described flow-activated sorting (FACS) based on *LacZ* (β-galactosidase) expression (Nolan *et al.*, 1988).

In the present embodiment, for example, putative control regions can be isolated from promoters whose activity can be detected in promoter-trap experiments after doses of 200-1,000 cGray of ionizing radiation. This level of radiation has been shown to induce the transcription of several genes (Boothman *et al.*, 1989; Woloschak *et al.*, 1990; Woloschak *et al.*, 1990; Hallahan *et al.*, 1989). Preferably x-ray and possibly gamma-ray radiation can be employed.

A human cell line known to contain a full chromosomal complement is used in these radiation studies, preferably either HL60 or HT1080. A minimum of 10^9 cells is infected with the promoter-trap retroviral vector that has been packaged through either Ψ-CRIP or GP+AM12 amphotropic lines. One available promoter-trap vector, pGgTKNeo*Lac*Zen(-) has the *LacZ* gene inserted 30 base pairs into the U3 LTR region replacing *his* in the original vectors (Dumenco *et al.*, 1989; Gansbacher *et al.*, 1990), and can be selected by constitutive expression of TKNeo (Reddy *et al.*, 1991). The first 8 codons of the *LacZ* gene are replaced by the insertion of a Shine-Delgarno/Kozak consensus sequence.

Another promoter-trap vector may be used, which is a derivative of a pGen-*geo* construct and that has the *β-geo* coding unit downstream of the consensus-spliced donor and acceptor elements isolated and characterized from adenovirus 5. The latter vector has the advantage of being active with integration anywhere downstream from the promoter, whereas the U3*LacZ* construct must integrate within 100-250 base pairs.

For promoter isolation, the target DNA which is juxtaposed to the 5′ end of the U3*LacZ* gene can be isolated by inverse PCR. For example, this can be accomplished by cutting the clone DNA with the restriction enzyme at *Cla*I sites in the LTR, recircularization by ligation at low concentration, enzymatic cleavage at the internal *Nhe*I site, and using a primer pair that specifically amplifies the linearized fragment, isolating the non-TkNeo containing amplimers. Due to the possibly large intervening distances with the splice donor *geo* trapped sequence, genomic DNA is cloned into a lambda genomic library that is plated on an *E. coli* strain permissive for amber suppressor codon utilization, thus insuring rescue only of phage containing the *SupF* marker that is inserted into the U3 of the pGen(-) vector. This method is based on the prediction that random integration into the genome in a large population of cells potentially places the marker gene downstream of and/or under control of a promoter which one wishes to isolate on the basis of specific attributes, induction with ionizing radiation, for example.

One week after mass infection, cells that express *LacZ* constitutively are sorted out from the non expressing cells by FACs run using the FDG fluorogenic *β*-galactosidase assay according to Nolan *t al.* (1988). This assay is a powerful technique to sort desired cells

and has been used to elucidate and sort cell lineages from early development (Krasnow *et al.*, 1991). All cells giving a signal in the autofluorescence and background range are ingated. This pre-screening step is repeated a week later to insure that the retrovirally infected cells do not stably express *LacZ*+ on a constitutive basis. Cells are irradiated in mass culture for the first time. Since gene induction by ionizing radiation occurs as an immediate to early response, separate cultures are set up and *LacZ*+ transient expression is monitored at 3 hour, 7 hour, 12 hour and 24 hour post-radiation treatment time points. For this, the cells are allowed to recover after radiation and at the specified time, removed for FACS analysis and sorted by ingating cells that express β-galactosidase activity above the cutoff range for background and autofluorescence. These first-round selected cells are then allowed to recover for another week and FACS selected for a second round for the *LacZ*+ phenotype after irradiation. After recovery for a week, a third cycle of selection takes place.

However, since cells have been irradiated on the order of several hundred cGy, permanent chromosome damage can be expected which might lead to a constitutive or high basal level of *LacZ*+ expression in some cells. For this reason, before the third cycle, the FACS pre-screen run is repeated, ingating only *LacZ* negative cells. These *LacZ*- cells are then irradiated as before for a third cycle of selection for cell-wise sorting of individual cells that have a high expression phenotype (suggesting good inducibility and tight regulation of whatever is controlling the *LacZ* gene). These cells are clonally expanded from pools of 10 cells and grown to approximately 10^7 cells, so that from 1 to 2 mg of genomic DNA can be isolated for inverse PCR or *SupF* marker rescue, and cloning can be performed to isolat the putative irradiation-inducible promoters or RRE

elements. The resultant clones or amplified DNA are subjected to automated sequencing for comparison to known cis-acting consensus sequence elements and for homology to promoter region(s) from other genes. These regions are selectively PCR amplified using appropriately designed primers that also have uniquely tailored restriction site tails, and are then cloned in promoterless CAT vectors for transient expression assays. Those constructs demonstrating a typically tightly regulated inducible response to ionizing radiation that do not involve the PKC pathway, and furthermore do not appear to involve other physiological regulatory pathways are ideal candidate promoters for the "indirect" targeting strategy.

FACS-FDG Sorting of Cells Based on LacZ Activity

The procedure is described in Nolan *et al.* (1988). Cell suspensions are loaded with the β-D-galactosidase substrate, fluorescein di-β-digalactopyranoside (FDG) (obtained from Molecular Probes, Inc, Eugene, OR, as Fluoreporter Kit) and stained by the two-color technique to separate live cells containing the *LacZ* gene constructs from dead cells for quantification and/or sorting. One hundred milliliters of cells in staining medium (PBS, 4% fetal bovine serum, 10 mM HEPES, pH 7.2) are suspended at approximately 10^7/ml and placed first on ice, then prewarmed to 37°C for 10 minutes. An equal volume of cell suspension is added to a 100 ml prewarmed working solution of FDG made by diluting the stock solution (20 mM FDG in 10% DMSO in water) 1:10 in dH_2O and then incubating at 37°C for exactly 1 minute. The loading is stopped by adding 1.8 ml ice-cold staining medium containing 1 mg/ml propidium iodide (PI) to the cell suspension, and storing on ice until the FACS run. The endogenous lysosomal galactosidase activity is inhibited by pretreatment with chloroquine (300 mM) 15 minutes before FDG loading in the staining medium.

Hydrolysis is arrested with 1 mM phenyl-β-D-galactoside (PETG) at the termination of the loading procedure to give the zero time control of background and autofluorescence, and at 10 minutes to detect *LacZ*+ cells. The cells counterstained with 1 mg/ml of propidium iodide are dead and not sorted. The dual-stained cells are analyzed with a Becton-Dickson FACS Star. Excitation is with the 488 nm line of the argon ion laser at a power of 15 mW, which excites both fluorescein and propidium iodide fluorescence. The cells are it-map-gated on forward and 90 light scatter which results in a preliminary separation of the viable cells. Red and green fluorescence are then ingated for analysis through a 520 +/- 10 nm interference filter; red fluorescence (propidium iodide) is analyzed at >630 nm with a long pass interference filter. The population of cells which are unstained by PI and with elevated green-fluorescence are live cells expressing the *LacZ* gene. Controls include cells not containing the construct, and cells incubated without substrate to detect autofluorescence.

Promoter Sequence Capture

The promoter region is captured by inverted PCR (Triglia *et al.*, 1988) using specific primers for the upper and lower strand flanking the U3 *Nhe*I site. This procedure is described (von Melchner *et al.*, 1990), and requires ligation under conditions favoring recircularization of *Cla*I digested genomic DNA, then linearization with *Nhe*I, inverted PCR amplification yielding both 5'genomic DNA and *Cla*I vector fragments containing the TKneo gene. After isolation of the amplimer containing 5' flanking genomic DNA, it is re-amplified under conditions for asymmetric PCR (Gyllenstein and Erlich, 1988). This is accomplished with an asymmetric ratio of the *Nhe*I primer pair so that linear amplification from the 5' end of U3 is obtained.

This product is subjected to automated did oxy sequencing using an ABI M370 unit. The sequence is analyzed using the University of Wisconsin Genetics Computer Group (UWGCG) program.

For pGen-geo vector promoter trap sequences, genomic DNA from clonal expansion of individual cells giving transient expression of *LacZ+* after irradiation is digested with *Eco*RI and ligated to Charon 3A arms which permit packaging of 0-12 kb (Blattner *et al.*, 1977). These are packaged according to Blattner *et al.*, (1978), and plated on DP50*Sup*F (c2098). Phage plaques that have picked up the *Sup*F marker are screened for the proviral insert by the procedure of Benton and Davis (1977), restriction mapped and the region upstream of the *LacZ/Cla*I site subcloned into *Eco*RI/*Cla*I digested pBluescript II KS. Phagemids are isolated after defective KO7 activation and used directly for automated sequencing.

* * *

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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(iii) TITLE OF INVENTION: PORE-FORMING AND SUPERANTIGEN ENCODING TOXIN CASSETTES FOR GENE THERAPY

(iv) NUMBER OF SEQUENCES: 8

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS/ASCII

(vii) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: Unknown
(B) FILING DATE: Concurrently herewith
(C) CLASSIFICATION: Unknown

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CGCCGCCACC ATGAGCGAGA AAAGCGAAGA A 31

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTATTGACT TAACTTGTAT ATAAATATAT 30

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 496 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

NTNCNKKKNN NNNAGTNCAN GANNNGGGNC NGGGCCCCCN TTGNGGCNGC NGGTANCGAT 60

AAGNTTGATG CGCCACCATG ATCGAGAAAA NCNAANAAAT AATGAAAAAN ATTTGCNAAA 120

AAAAGTCTGA ATTGCAGGGA ACAGCTTTAG GCAATCTTAA ACAAATCTAT TATTACAATG 180

AAAAAGCTAA AACTGAAAAT AAAGAGAGTC ACGATCAATT TTTACAGCAT ACTATATTGT 240

TTAAAGGCTT TTTTACAGAT CATTCGTGGT ATAACGATTT ATTAGTAGAT TTTGATTCAA 300

AGGATATTGT TGATAAATAT AAAGGGAAAA AAGTAGGCTT GTATGGTGCT TATTATGGTT 360

ATCCAATGTG CGGGTGGTAC ACCAAACCAA ANCAGCTTGT ATGTATGGTG GTGTACCGTT 420

ACATGGNTAA TAATCGNTTG GCCGNGGGGG ANAAAAGTGC CGNTCCAATT TATGGCTNGG 480

CCGGGTAACC AAANTC 496

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 498 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

NTNCNKKKNN NNNAGTNCAN GANNNGGGNC NGGGCCCCCN TTGNGGCNGC NGGTANCGAT 60

AAGNTTGATC GCCGCCACCA TGATCGAGAA AATCGAATAA ATAATGAAAA AGATTTGCNA 120

AAAAAAGTCT GAATTGCAGG GAACAGCTTT AGGCAATCTT AAACAAATCT ATTATTACAA 180

TGAAAAAGCT AAAACTGAAA ATAAAGAGAG TCACGATCAA TTTTTACAGC ATACTATATT 240

GTTTAAAGGC TTTTTTACAG ATCATTCGTG GTATAACGAT TTATTAGTAG ATTTTGATTC 300

AAAGGATATT GTTGATAAAT ATAAAGGGAA AAAAGTAGGC TTGTATGGTG CTTATTATGG 360

TTATCCAATG TGCGGGTGGT ACACCAAACC AAANCAGCTT GTATGTATGG TGGTGTACCG 420

TTACATGGNT AATAATCGNT TGGCCGNGGG GGANAAAAGT GCCGNTCCAA TTTATGGCTN 480

GGCCGGGTAA CCAAANTC 498

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTTTCCAGG GTGCCCCAAG G 21

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGAGAGGAGA CCCTCC 16

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CAAGATGGAT TGCACGCAGG 20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGCAAGGTG AGATGACAGG 20

CLAIMS

1. A DNA segment comprising a superantigen encoding gene cassette positioned under the control of a promoter element expressible in a mammalian cell, the gene and promoter being adapted for expression in a eukaryotic cell.

2. A DNA segment according to claim 1 which further comprises a selectable marker gene.

3. A DNA segment according to claim 1 wherein the gene includes a transcription start site, a eukaryotic ribosome binding site, a transcription termination site and a poly-adenylation site.

4. A DNA segment according to claim 1 wherein the promoter element is an inducible promoter element, controllable by an exogenously or endogenously applied agent.

5. A DNA segment according to claim 4 wherein the promoter is inducible by a metal cation, heat or ionizing radiation.

6. A DNA segment according to claim 5 wherein the promoter is induced by cadmium ions.

7. A DNA segment according to claim 6 wherein the inducible promoter is a modified metallothion in promoter.

8. A DNA segment according to claim 1 in which the superantigen encoding region encodes a product which permeabilizes the cell membrane and is cytotoxic to the cell.

9. A DNA segment according to claim 8 wherein said superantigen encoding region encodes *Staphylococcus* enterotoxin A, B, C1, C2, C3, D, E or F; staphylococcal enterotoxin-like streptococcal toxins, *Mycoplasma arthriditis* toxin or mammary tumor virus superantigen, Mtv-7.

10. A DNA segment according to claim 9 in which said superantigen encoding region encodes the *Staphylococcus* enterotoxin A.

11. A DNA segment according to claim 2 in which the selectable marker is an antibiotic resistance marker.

12. A DNA segment according to claim 11 in which the antibiotic resistance marker confers resistance to neomycin.

13. A DNA segment according to claim 2 in which the selectable marker conf rs resistance to hygromycin B,

puromycin, bleomycin (phleomycin) histadiol, gpt, β-gal or chloramphenicol acetyl transferase.

14. The DNA segment of claim 1 further defined as a recombinant vector.

15. A DNA segment according to claim 14 in which the vector is a plasmid.

16. A DNA segment according to claim 14 in which the vector is a viral vector.

17. A DNA segment according to claim 16 in which the vector is a retroviral vector.

18. A DNA segment according to claim 17 in which the retroviral vector is of the pBABE series.

19. A DNA segment according to claim 17 in which the retroviral vector is pGEN(-) or pHMB.

20. A DNA segment according to claim 16 in which the vector is an adeno-associated virus.

21. A method of treating human cancer patients by insertion of the DNA segment of claim 16 into the cells of a human tumor and inducing expr ssion of the said sup rantigen encoding gen in the said human tumor cells.

22. A meth d according to claim 21 in which the superantigen gene expression is induced by cadmium ions, heat or ionizing radiation.

23. A method according to claim 21 wherein the said superantigen is *Staphylococcus* enterotoxin A, B, C1, C2, C3, D, E or F; staphylococcal enterotoxin-like streptococcal toxins, *Mycoplasma arthriditis* toxin or mammary tumor virus superantigen, Mtv-7.

24. A method according to claim 23 wherein said superantigen gene encodes *Staphylococcus* enterotoxin A.

START
MET
5' CGCCGCCACCATG AGC GAG AAA AGC GAA GAA
Shine-Dalgarno
Complement SEA at 1st Amino Acid serine
3' TAT ATA AAT ATA TGT TCA ATT CAGTTATCG
Complement 3' end SEA
STOP

FIGURE 1A

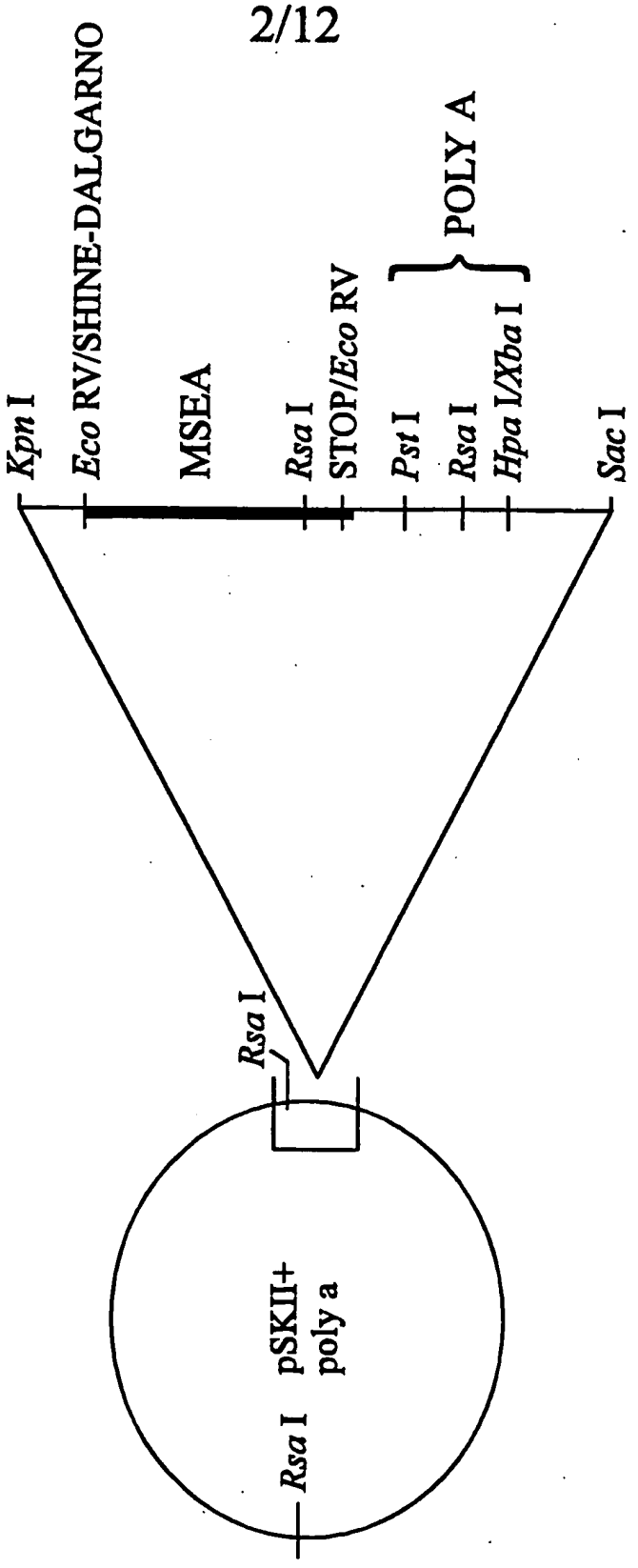
Kpn I
Eco RV/SHINE-DALGARNO
MSEA
Rsa I
STOP/Eco RV
Pst I
Rsa I
Hpa I/Xba I
POLY A
Sac I
Rsa I
pSKII+
poly a
Rsa I

FIGURE 1B

```
NTNCNKKKNN NNNAGTNCAN GANNNGGGNC NGGGCCCCCN TTGNGGCNGC NGGTANCGAT   60
AAGNTTGATG CGCCACCATG ATCGAGAAAA NCNAANAAAT AATGAAAAAN ATTTGCNAAA  120
AAAAGTCTGA ATTGCAGGGA ACAGCTTTAG GCAATCTTAA ACAAATCTAT TATTACAATG  180
AAAAAGCTAA AACTGAAAAT AAAGAGAGTC ACGATCAATT TTTACAGCAT ACTATATTGT  240
TTAAAGGCTT TTTTACAGAT CATTCGTGGT ATAACGATTT ATTAGTAGAT TTTGATTCAA  300
AGGATATTGT TGATAAATAT AAAGGGAAAA AAGTAGGCTT GTATGGTGCT TATTATGGTT  360
ATCCAATGTG CGGGTGGTAC ACCAAACCAA ANCAGCTTGT ATGTATGGTG GTGTACCGTT  420
ACATGGNTAA TAATCGNTTG GCCGNGGGGG ANAAAAGTGC CGNTCCAATT TATGGCTNGG  480
CCGGGTAACC AAANTC                                                  496
```

FIGURE 2A

```
NTNCNKKKNN NNNAGTNCAN GANNNGGGNC NGGGCCCCCN TTGNGGCNGC NGGTANCGAT    60
AAGNTTGATC GCCGCCACCA TGATCGAGAA AATCGAATAA ATAATGAAAA AGATTTGCNA   120
AAAAAAGTCT GAATTGCAGG GAACAGCTTT AGGCAATCTT AAACAAATCT ATTATTACAA   180
TGAAAAAGCT AAAACTGAAA ATAAAGAGAG TCACGATCAA TTTTTACAGC ATACTATATT   240
GTTTAAAGGC TTTTTTACAG ATCATTCGTG GTATAACGAT TTATTAGTAG ATTTTGATTC   300
AAAGGATATT GTTGATAAAT ATAAAGGGAA AAAAGTAGGC TTGTATGGTG CTTATTATGG   360
TTATCCAATG TGCGGGTGGT ACACCAAACC AAANCAGCTT GTATGTATGG TGGTGTACCG   420
TTACATGGNT AATAATCGNT TGGCCGNGGG GGANAAAAGT GCCGNTCCAA TTTATGGCTN   480
GGCCGGGTAA CCAAANTC                                                 498
```

FIGURE 2B

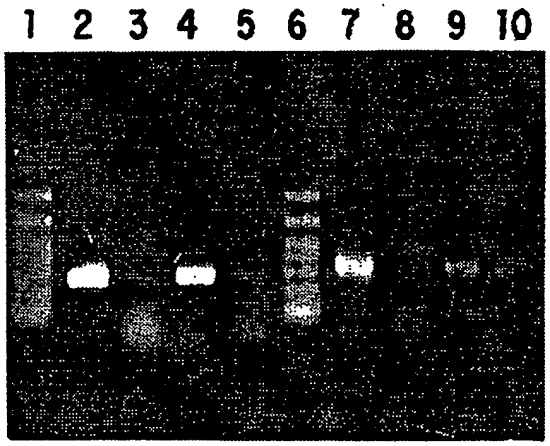

FIGURE 3

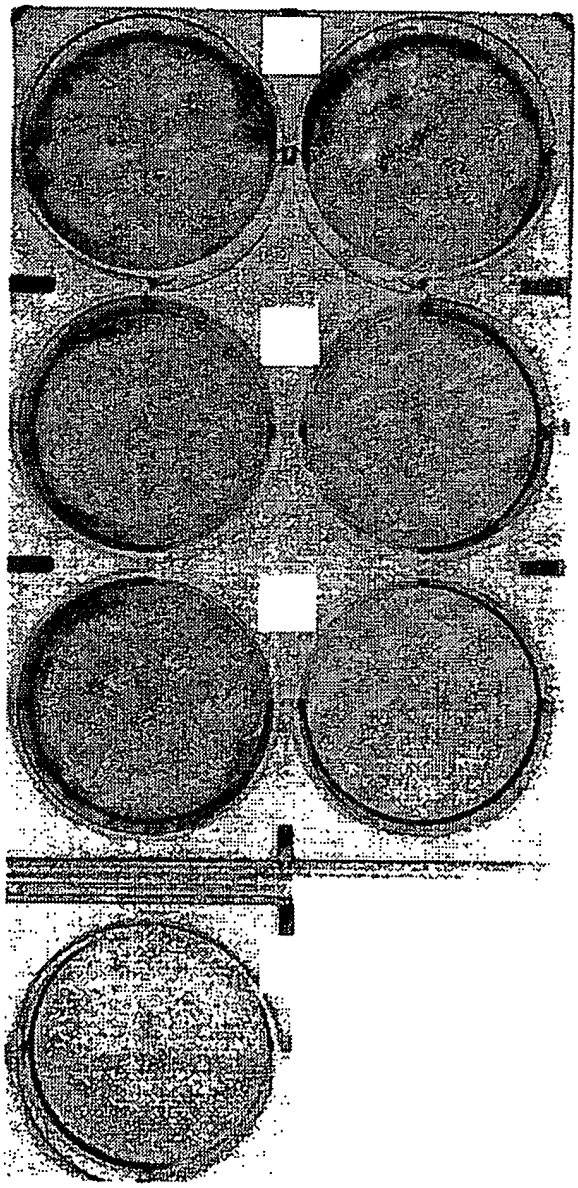

FIGURE 4

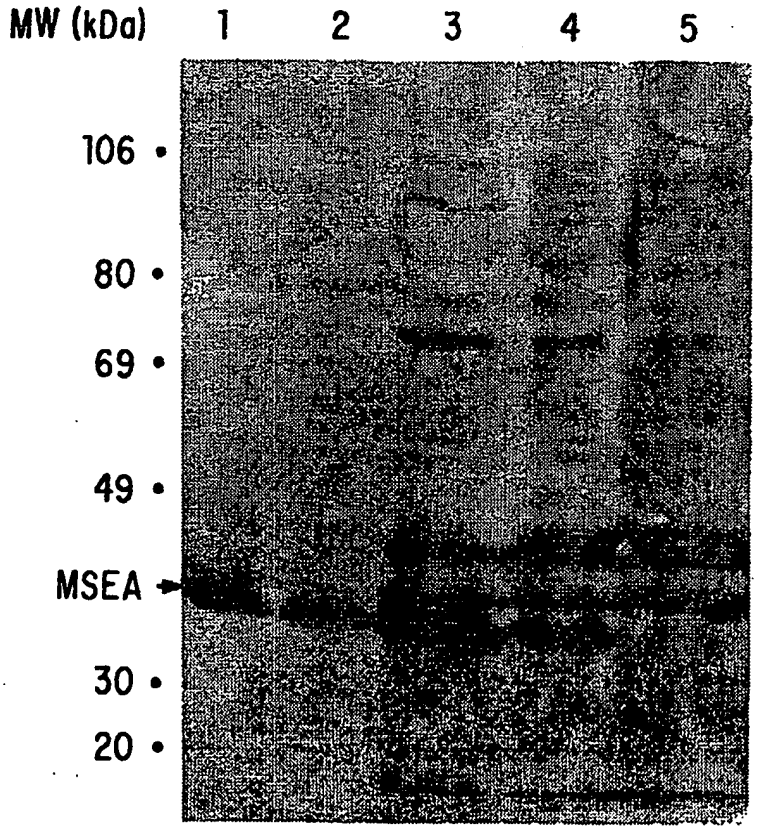

FIGURE 5

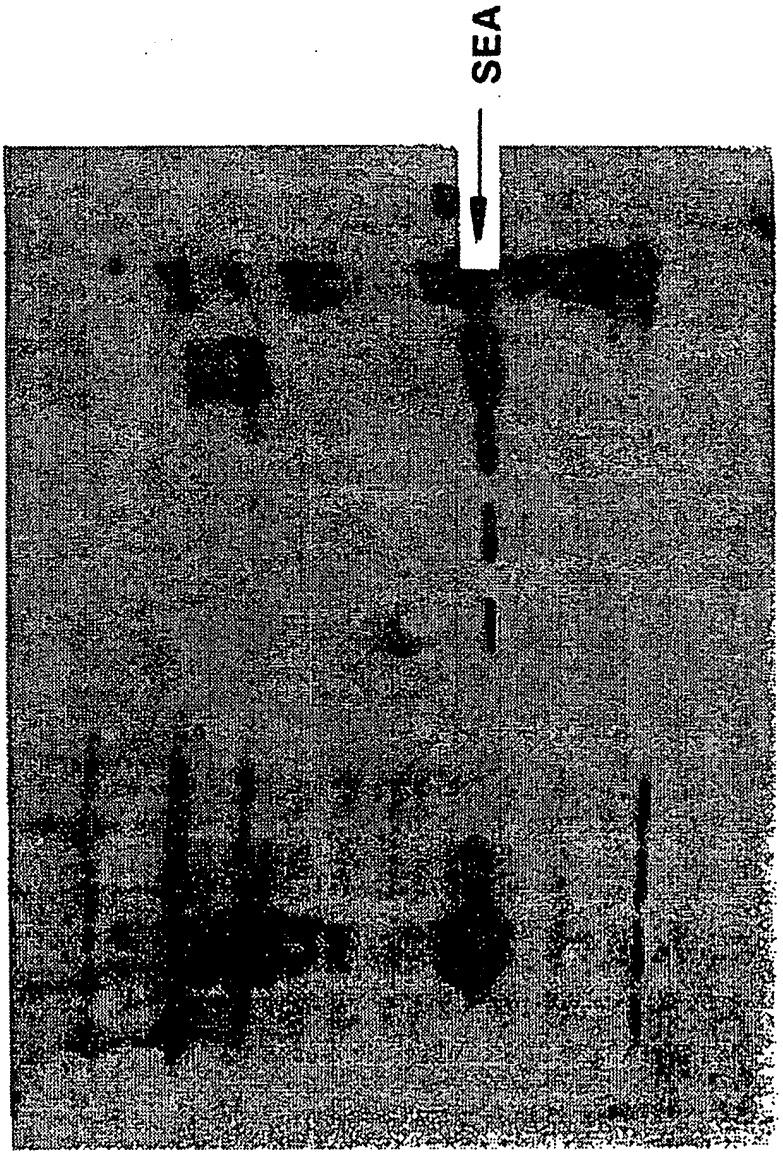

FIGURE 6

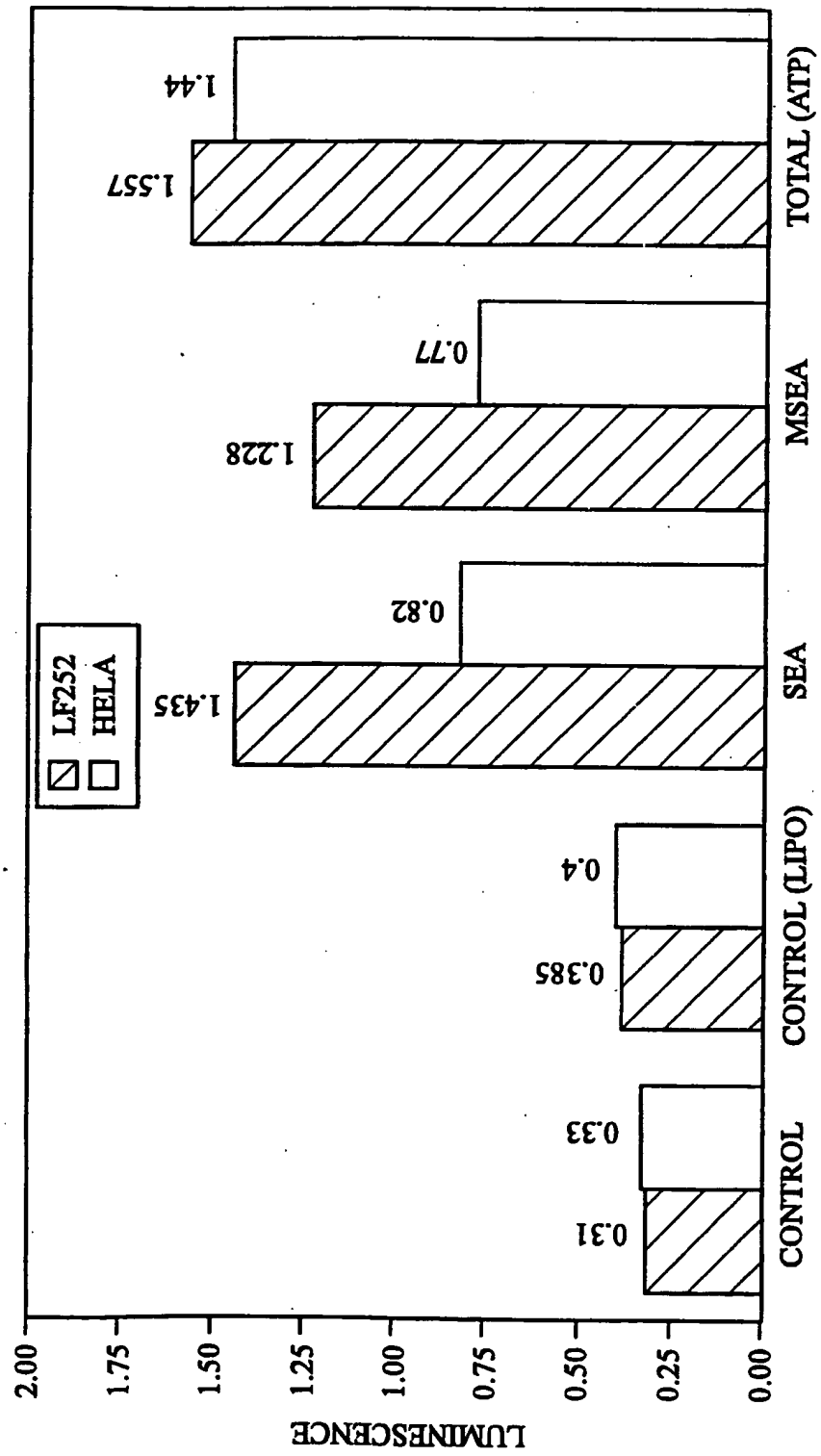
LUMINESCENCE
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
LF252
HELA
1.435
0.82
1.228
0.77
1.557
1.44
0.385
0.4
0.31
0.33
SEA
MSEA
TOTAL (ATP)
CONTROL (LIPO)
CONTROL

FIGURE 7

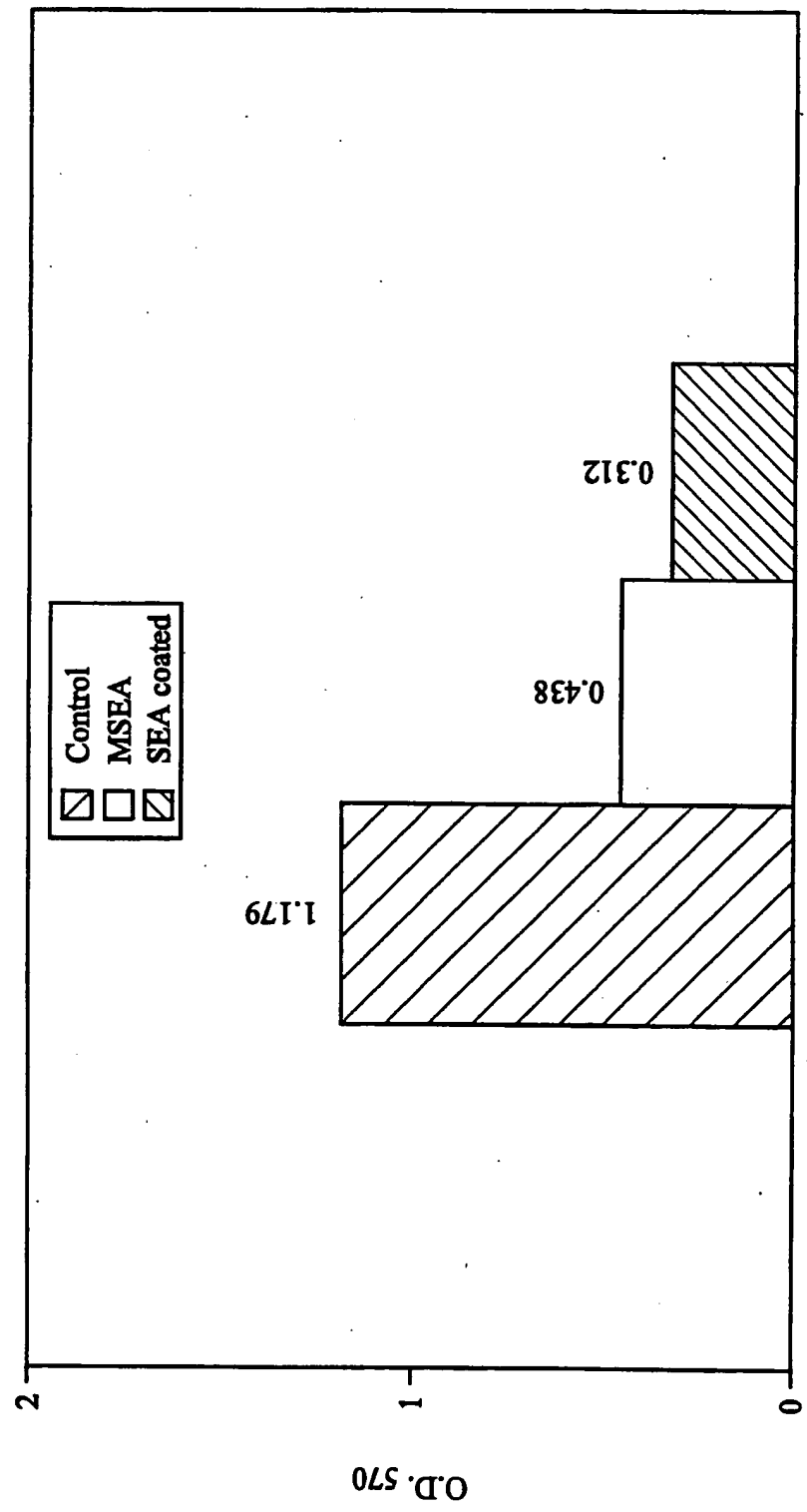
Control
MSEA
SEA coated
0.312
0.438
1.179
TREATMENTS
2
1
0
O.D. 570

FIGURE 8

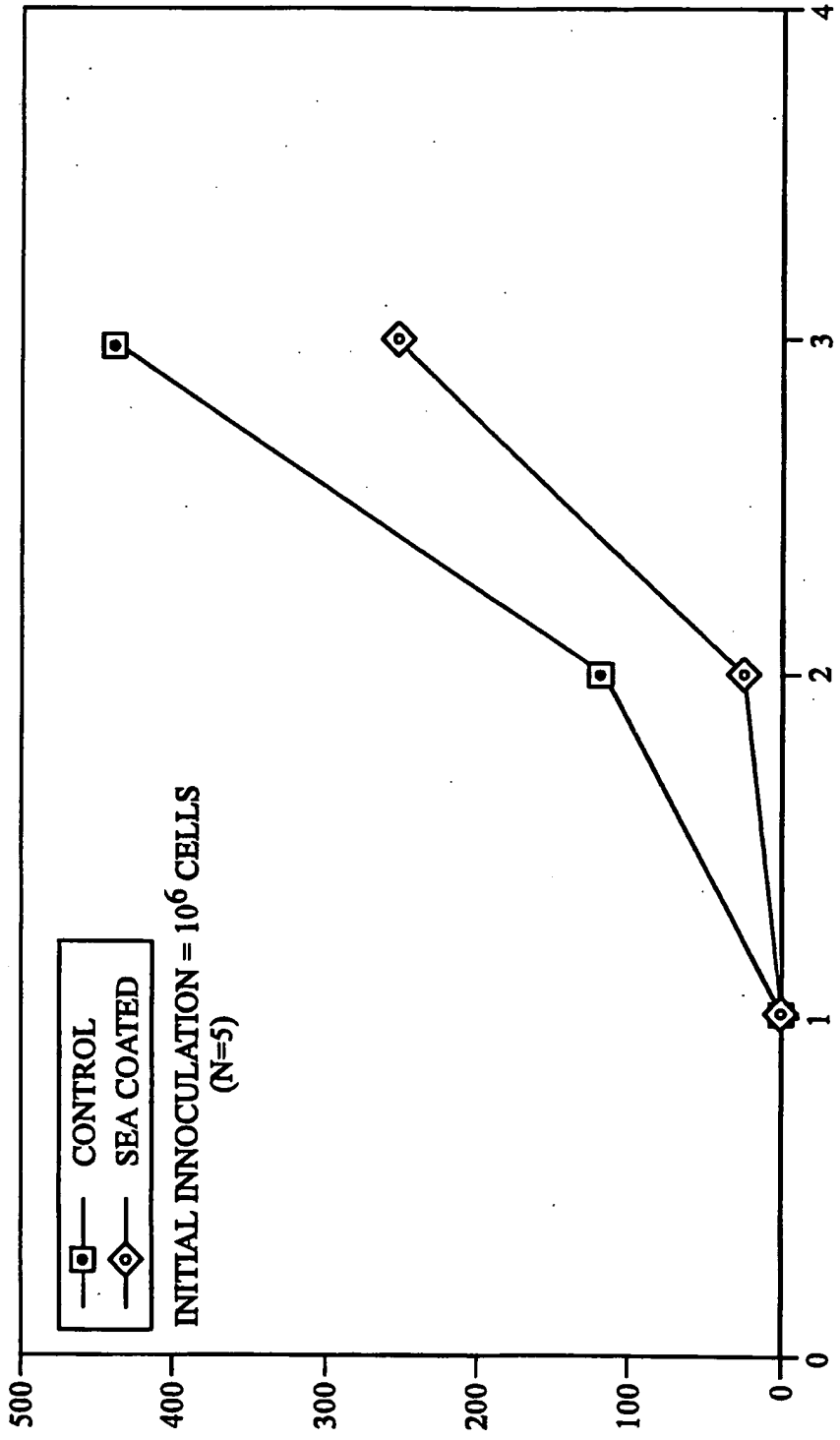
TUMOR GROWTH (mm^2)
500
400
300
200
100
0
0
1
2
3
4
DAYS (x10)
CONTROL
SEA COATED
INITIAL INNOCULATION = 10^6 CELLS
(N=5)

FIGURE 9

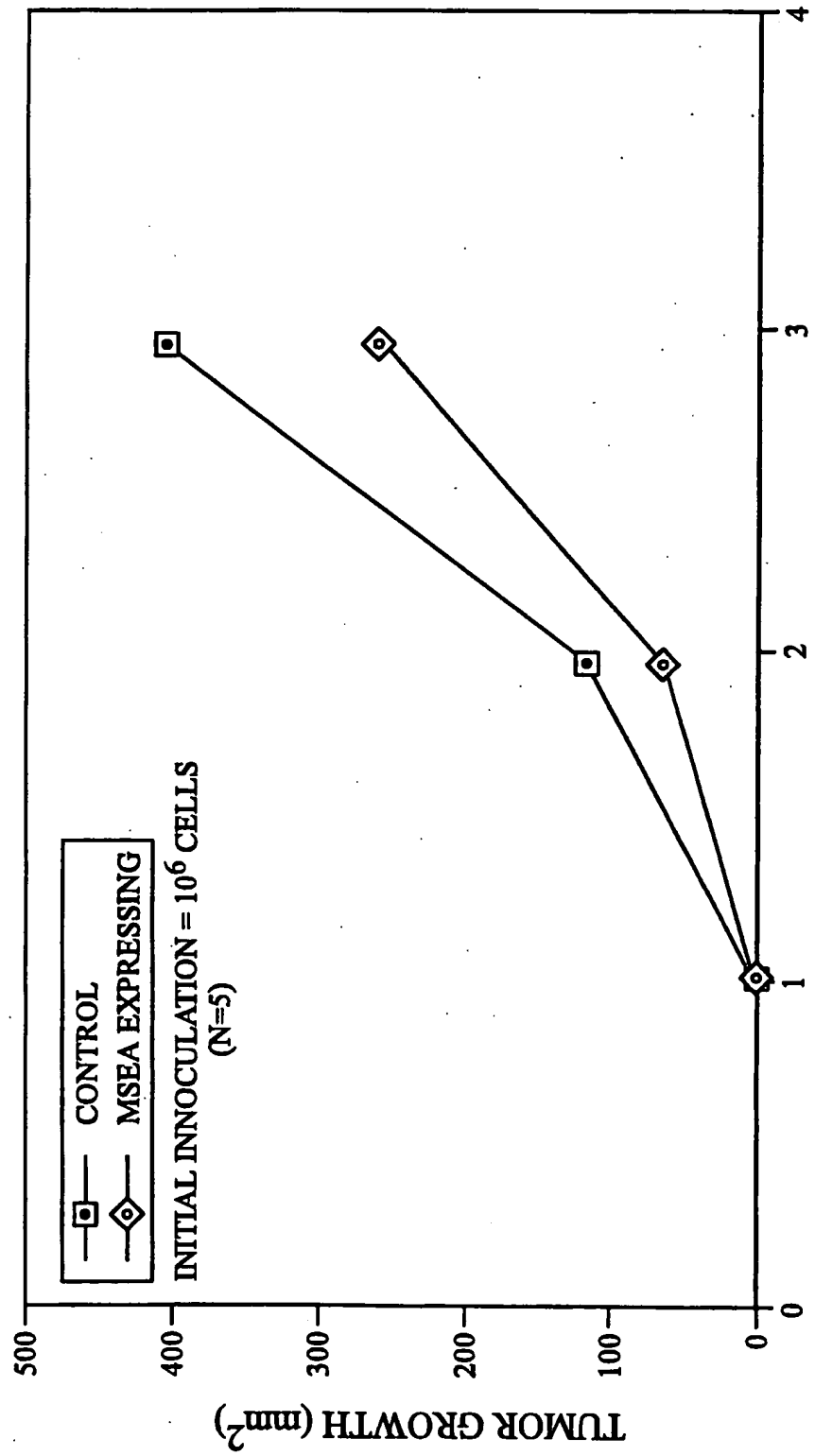
TUMOR GROWTH (mm^2)
500
400
300
200
100
0
0
1
2
3
4
DAYS (x10)
CONTROL
MSEA EXPRESSING
INITIAL INNOCULATION = 10^6 CELLS
(N=5)

FIGURE 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07091

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 48/00
US CL :514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Volume 17, No. 10, issued 1989, R.G. Hawley et al., "An improved retroviral vector for gene transfer into undifferentiated cells", page 4001, see entire article.	16, 17, 19
Y	Journal of Bacteriology, Volume 170, No. 1, issued January 1988, M.J. Betley et al., "Nucleotide sequence of the Type A Staphylococcal Enterotoxin Gene", pages 34-41, see entire article.	1-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
29 JULY 1994

Date of mailing of the international search report
16 AUG 1994

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07091

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 81, issued October 1984, P. L. Hermonat et al., "Use of adeno - associated virus as a mammalian DNA cloning vector: Transduction of neomycin resistance into mammalian tissue culture cells", pages 6466-6470, see entire article.	16, 20
Y	Nucleic Acids Research, Volume 18, No. 12, issued 1990, J. P. Morgenstern et al., "Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line", pages 3587-3596, see entire article.	16-18
Y	Proceedings of the National Academy of Sciences, Volume 88, issued October 1991, M. Dohlsten et al., "Monoclonal antibody-targeted superantigens: A different class of anti-tumor agents", pages 9287-9291, see entire article.	1-15, 21-24
Y	Proceedings of the National Academy of Sciences, Volume 87, issued June 1990, L. Ercolani et al., "Membrane localization of the pertussis toxin-sensitive G-protein subunits αi-2 and αi-3 and expression of a metallothionein-αi-2fusion gene in LLC-PK1 cells", pages 4635-4639, see entire article.	1-15